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3-[(2-Methyl-1,3-thiazol-4-yl)ethynyl]-pyridine: A Potent and Highly Selective Metabotropic Glutamate Subtype 5 Receptor Antagonist with Anxiolytic Activity

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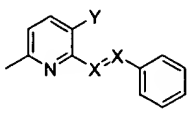
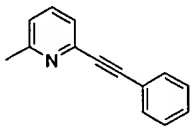
Abstract: 2-Methyl-6-(phenylethynyl)pyridine (**3**), a potent noncompetitive mGlu5 receptor antagonist widely used to characterize the pharmacology of mGlu5 receptors, suffers from a number of shortcomings as a therapeutic agent, including off-target activity and poor aqueous solubility. Seeking to improve the properties of **3** led to the synthesis of compound **9**, a highly selective mGlu5 receptor antagonist that is 5-fold more potent than **3** in the rat fear-potentiated startle model of anxiety.

Introduction. Glutamate is the principal excitatory transmitter in the central nervous system acting through ionotropic glutamate receptors; however, it also plays a major role in activating modulatory pathways through G-protein-coupled metabotropic glutamate (mGlu) receptors.[1,2] Group I mGlu receptors include the mGlu1 and mGlu5 subtypes, which are coupled to stimulation of phospholipase C resulting in phosphoinositide hydrolysis and elevation of intracellular Ca^{2+} levels ($[Ca^{2+}]_i$).[3,4] Excessive activation of mGlu5 receptors has been implicated in several diseases, and selective mGlu5 receptor antagonists may be of therapeutic benefit in the treatment of various pain states,[5] psychiatric disorders such as anxiety and depression,[6−11] and other neurological impairments such as drug addiction and drug withdrawal.[12]

The discovery in these laboratories of the first subtype selective, noncompetitive mGlu5 receptor antagonists, **1** and **2**,[13] led to the subsequent identification of 2-methyl-6-(phenylethynyl)pyridine (MPEP, **3**), a structurally related compound with similar selectivity but improved in vitro potency at mGlu5 receptors.[14] These



1 X=N, Y=OH
2 X=CH, Y=H



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pharmacological tools have led to a plethora of research to understand the role of mGlu5 receptors in the brain and nervous system and hence the viability of mGlu5 receptors as a molecular therapeutic target.[15]

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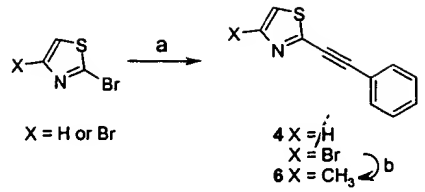
Scheme 1[a]

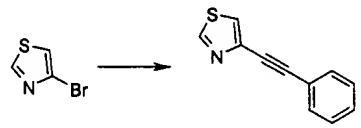


X = H or Br

4 X = H
X = Br
6 X = CH_3

[a] Reagents and conditions: (a) for X = H; phenylacetylene, $PdCl_2(PPh_3)_2$, CuI, NEt_3, DME, 80 °C, 16 h, (60%); for X = Br; phenylacetylene, $Pd(PPh_3)_4$, CuI, NEt_3, DME, 80 °C, 18 h, (86%); (b) Me_4Sn, $Pd_2(dba)_3$, $P(tBu)_3$, CsF, 1,4-dioxane, 100 °C, 5 h, (17%).

Scheme 2[a]



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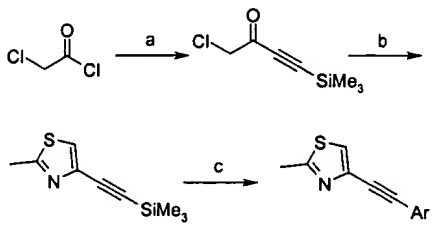
[a] Reagents and conditions: phenylacetylene, $Pd(PPh_3)_4$, CuI, NEt_3, DME, 80 °C, 5 h, (77%).

Scheme 3[a]



7 Ar = Ph
8 Ar = 2-pyridyl
9 Ar = 3-pyridyl
10 Ar = 4-pyridyl

[a] Reagents and conditions: (a) BTMSA, $AlCl_3$, CH_2Cl_2, 0 °C, 2 h and then 25 °C, 1 h, (54%); (b) thioacetamide, DMF, 25 °C, 16 h, (72%); (c) for Ar = Ph; iodobenzene, $PdCl_2(PPh_3)_2$, PPh_3, CuI, NEt_3, TBAF, Bu_4NI, DMF, 70 °C, 36 h, (73%); for Ar = 2-py; 2-bromopyridine, $PdCl_2(PPh_3)_2$, CuI, NEt_3, TBAF, DMF, 70 °C, 30 min, (73%); for Ar = 3-py; 3-bromopyridine, Pd $(PPh_3)_4$, CuI, NEt_3, TBAF, DME, 70 °C, 26 h, (65%); for Ar = 4-py; 4-bromopyridine hydrochloride, $Pd(PPh_3)_4$, CuI, NEt_3, TBAF, DME, 70 °C, 32 h, (75%).

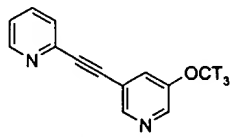


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Figure 1. [^{3}H]-3-methoxy-5-(pyridin-2-ylethynyl)pyridine.

While compound **3** has proven to be a useful tool for both in vitro and in vivo studies, it has a number of drawbacks as a drug. First, compound **3** is not completely selective for mGlu5 receptors. For example, it has been shown to block *N*-methyl-D-aspartate (NMDA) receptors,[16,17] albeit at high concentrations, and our own studies suggest the presence of other off-target activities of **3**. Second, the compound is poorly water soluble, as indicated by a log *D* value of 3.5, which would limit solubility in CSF and very likely reduce in vivo efficacy.

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Table 1. In Vitro Potency Data for mGlu5 Receptor Antagonists[a]

	A	B	hmGlu5 Ca^{2+} Flux IC_{50} (nM)[b]	mGlu5 K_i (nM)[c]	logD[d]
3		Ph	2 (0.25; 5)	12 (0.40; 9)	3.5
4		Ph	81 (0.22; 12)	153 (0.21; 3)	3.2
5		Ph	97 (0.36; 3)	142 (0.14; 3)	2.6
6		Ph	13 (0.05; 3)	32 (0.11; 3)	3.6
7		Ph	6 (0.74; 5)	6 (0.45; 3)	3.3
8		2-Py	53 (0.05; 4)	48 (0.12; 3)	1.9
9		3-Py	5 (0.40; 3)	16 (0.20; 4)	2.1
10		4-Py	122 (0.16; 4)	214 (0.08; 3)	2.1

[a] Data are presented as the geometric mean followed in parentheses by the standard deviation and the number of replicates. [b] Ca^{2+} flux assay using glutamate as agonist.[13,19,20] [c] Displacement by test compounds of [^{3}H]-3-methoxy-5-(pyridin-2-ylethynyl)pyridine (**11**) bound to rat cortical membranes.[21] [d] See ref 18.

Table 2. In Vitro Selectivity Data for mGlu5 Receptor Antagonists **3** and **9**

	mGlu1[a] IC_{50} (μM)	NR2B[b] IC_{50} (μM)	MAO_A[c] IC_{50} (μM)
3	>100	18	8 (6, 13)
9	>100	>300	30 (17, 40)

[a] Ca^{2+} flux assay, tested as agonist and antagonist. [b] Ca^{2+} flux assay; data generated from a six-point CRC, n = 8–22 cells/concentration tested. [c] Data are presented as the geometric mean IC_{50} followed in parentheses by the upper and lower SE (n = 3).

We therefore sought mGlu5 receptor antagonists with improved selectivity, a lower log D value, and good efficacy in vivo. With this goal in mind, we set out to explore the SAR of **3** in order to identify a compound possessing all of these desirable properties.

Medicinal Chemistry and SAR. The Sonogoshira cross-coupling methodology employed for the synthesis of the alkyne derivatives described herein is summarized in Schemes 1–3 (yields are not optimized). Two in vitro assays were employed in the initial research phase in addition to the determination of log D for compounds using an HPLC method.[18] The functional potency of compounds was assessed using an automated assay employing Ltk cells stably expressing human recombinant mGlu5 receptors. This cell-based assay measures changes in cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_i$) by fluorescence detection using the Ca^{2+}-sensitive dye fura-2.[13,19,20] Binding to native mGlu5 receptors in vitro was determined by measuring the displacement by test compounds of [^{3}H]-3-methoxy-5-(pyridin-2-ylethynyl)pyridine (**11**, Figure 1) from rat cortical membranes.[21]

The 1,3-thiazole ring is a classical isostere of pyridine, and therefore, a logical first step was to prepare analogues in which the pyridyl ring (A) in **3** was replaced with a thiazole moiety. Replacement of the 6-methylpyridine moiety in **3** with 2- or 4-thiazolyl units, as in **4** and **5**, gave compounds with reduced in vitro potency at the mGlu5 receptor (Table 1). By analogy with **3**, introduction of a methyl group adjacent to the thiazole nitrogen in **4** and **5** gave compounds **6** and **7** with greatly enhanced in vitro potency at the mGlu5 receptor. In particular, 2-methyl-4-(phenylethynyl)-1,3-thiazole (**7**) inhibited mGlu5 recepor mediated Ca^{2+} flux with IC_{50} = 6 nM and K_i = 6 nM in the binding assay, which is in the same potency range as compound **3** in these assays. However, log D values (Table 1) indicated that both **3** (log D = 3.5) and **7** (log D = 3.3) are lipophilic molecules. It was hypothesized that introduction of a second basic nitrogen into the molecular framework of **7** would reduce the log D and increase aqueous solubility. A nitrogen scan in the phenyl ring (B) of compound **7** demonstrated that, while the 2-and 4-pyridyl isomers (**8** and **10**) were less potent than **7**, the 3-pyridyl isomer (**9**) was potent in vitro (IC_{50} = 5 nM in the Ca^{2+} flux assay, K_i = 16 nM). Furthermore, 3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine (MTEP, **9**) has a log D value of 2.1 compared with 3.5 for **3** and 3.3 for **7** (Table 1). On the basis of these data, **9** was selected for further in vitro and in vivo evaluation.

In Vitro Profiles of Compounds 3 and 9. To identify any off-target activities, **3** and **9** were profiled extensively against a battery of in vitro assays. Experiments were performed initially at a drug concentration of 10 μM. For assays in which inhibition was detected at the single dose, concentration–response curves were generated. Like **3**, compound **9** is highly selective for the mGlu5 receptor over the mGlu1 receptor (Table 2). Additionally **9** has no effect when tested against other mGlu receptor subtypes (e.g., mGlu1, mGlu2, mGlu7) or against ionotropic glutamate receptors, including AMPA and kainate subtypes (e.g., Glu1, Glu3, Glu5, Glu6). It should be noted that **3** and **9** were not tested against glutamate transporters or against enzymes for which glutamate is a substrate. Since it had been reported that **3** is an antagonist of NMDA NR2B-containing receptors in vitro,[16,17] **3** and **9** were tested for their ability to block NMDA/glycine-evoked increases in intracellular calcium in a cell line stably expressing recombinant human NMDA receptors. In this assay, **3** inhibited NR1A/2B receptors (IC_{50} = 18 μM, Table 2) while **9** produced only 19% inhibition at a concentration of 300 μM. Further profiling revealed that **3** displaced [^{125}I]methyl (1*R*,2*S*,3*S*,5*S*)-3-(4-iodophenyl)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylate ([^{125}I]RTI-55) from MAO_A with IC_{50} = 8 μM compared with IC_{50} = 30 μM

Table 3. In Vivo Data for mGlu5 Receptor Antagonists **3** and **9**[a]

	Occ ED_{50}[b,c] (mg/kg ip)	plasma levels[d,e] (μM)	brain levels[d,e] (μM)	CSF levels[d,f] (μM)	FPS ED_{50}[b,g] (mg/kg ip)
3	2.1 (1.1, 3.4)	0.39 ± 0.02 (7)	0.83 ± 0.05 (7)	0.21 ± 0.3 (5)	5 (3.7, 6.66)
9	1 (0.6, 1.2)	1.2 ± 0.2 (6)	1.4 ± 0.2 (6)	1.0 ± 0.27 (5)	1 (0.65, 2.0)

[a] All in vivo measurements taken at 1 h after administration. [b] Data presented as mean followed in parentheses by ED_{50} values from individual experiments (95% confidence interval). [c] n = 4–6 rats/group. [d] Data presented as mean ± SEM (n), where n = number of rats/group. [e] Dose = 3 mg/kg ip. [f] Dose = 30 mg/kg po. [g] n = 8 rats/group.

for **9**. Together, these results indicate that **9** exhibits fewer off-target effects than **3** and thus greater specificity for the mGlu5 receptor.

In Vivo Profiles of Compounds 3 and 9. To evaluate the brain penetration of **3** and **9** and to correlate affinity at the mGlu5 receptor with in vivo efficacy, an in vivo receptor occupancy assay was employed.[22] Briefly, at time zero rats were dosed with the test compound intraperitoneally and at 59 min. [^{3}H]-3-methoxy-5-(pyridin-2-ylethynyl)pyridine (**11**)[21] was administered via tail vein injection. One minute later, the animals were sacrificed and brain binding was measured. With this paradigm, dose−response relationships were determined for binding to the mGlu5 receptor in vivo and it was found that **9** is twice as potent as **3** in this assay (Table 3). In other experiments, plasma, brain (hippocampus), and CSF levels for **3** and **9** were measured following dosing in rats. Interestingly, while both **3** and **9** exhibited similar drug levels in the hippocampus (Table 3), the concentration of **9** in CSF (1 μM) was approximately 5-fold higher than the concentration of **3** (0.21 μM). This may be a consequence of the lower log *D* value for **9** and therefore greater aqueous solubility compared with **3** (Table 1).

There is growing evidence of a role for mGlu5 receptors in the modulation of mood disorders including depression and anxiety.[6-11] For example, **3** is reported to be active in the rodent Geller−Seifter model,[6,7] ultrasonic vocalization,[6] elevated plus maze,[7,9] social exploration test,[7] marble burying test,[7] conflict drinking test,[9] and four-plate test.[9] Compound **3** also reduces stress-induced hyperthermia in mice.[8] Recently **3** was also shown to block fear-conditioning in rats as determined in the fear-potentiated startle (FPS) model of anxiety.[6,11] The FPS model was therefore selected to assess the relative potencies of **3** and **9** in a rodent model of anxiety. Compounds **3** and **9** were administered intraperitoneally to rats, and both compounds were found to block the expression of fear in this paradigm. However, the ED_{50} for **9** was calculated to be 1 mg/kg compared with 5 mg/kg for **3** (Table 3), indicating that **9** is 5-fold more potent than **3** in this animal model.

Conclusion. Exploration of the SAR around **3** resulted in the discovery of compound **9**, a potent and selective mGlu5 receptor antagonist with fewer off-target effects than **3**. Furthermore, **9** is more potent than **3** in vivo (rats) in both a receptor occupancy assay and in the fear-potentiated startle model of anxiety. Further details of the SAR and pharmacological profile of **9** and analogues will be reported in due course.

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Supporting Information Available: Experimental details for the preparation of compounds **4**−**10** and log *D* determination using HPLC. This material is available free of charge via the Internet at http://pubs.acs.org.

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Anxiolytic-like activity of the mGluR5 antagonist MPEP A comparison with diazepam and buspirone

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Abstract

The selective and systemically active antagonist for the metabotropic glutamate receptor subtype 5 (mGluR5), 2-methyl-6-(phenylethynyl)pyridine (MPEP) was shown to display anxiolytic-like activity in a number of unconditioned assays of stress and anxiety (elevated plus maze, shock probe burying, marble burying, social interaction, and stress-induced hyperthermia) in rodents. In this report, we extend these observations found using unconditioned models of anxiety to include three models of conditioned anxiety, comparing the activity of MPEP to the clinically used anxiolytics, diazepam, and buspirone. MPEP and diazepam, but not buspirone, showed anxiolytic-like activity in the fear-potentiated startle (FPS) model. In a conditioned ultrasonic vocalization (USV) procedure, MPEP, diazepam, and buspirone reduced vocalizations to a similar degree. In the modified Geller–Seifter procedure, MPEP, diazepam, and buspirone displayed statistically significant anxiolytic-like activity, increasing the number of punished responses. Thus, these findings confirm and extend previous reports that MPEP exhibits anxiolytic-like activity in rats, and suggests that development of mGluR5 antagonists may provide a novel approach to treating anxiety disorders.

Keywords: MPEP; Anxiety; Ultrasonic vocalizations; Rat

1. Introduction

Benzodiazepines are the most commonly prescribed anxiolytic drugs, being efficacious against a spectrum of anxiety disorders. However, there are issues with addiction, tolerance, and dependence/withdrawal, as well as adverse side effects that include sedation, cognitive and psychomotor impairment, and anterograde amnesia. The other major classes of compounds used to treat anxiety are selective serotonin reuptake inhibitors (SSRIs) and the 5HT-1A partial agonist, buspirone. However, both classes of compounds have a slow onset of action (4–6 weeks) and their own side profiles. There is therefore a need for anxiolytics that show a rapid onset of action and an efficacy similar to benzodiazepines, with a low abuse potential and minimal impairment of cognition and motor skills. Since benzodiazepines act to increase inhibitory GABAergic transmission, an alternate approach to achieving the same end point might be to reduce excitatory glutamatergic neurotransmission.

Glutamate is the main excitatory neurotransmitter in the brain, acting through ionotropic and metabotropic (mGlu) receptor subtypes (Monaghan et al., 1989; Conn and Pin, 1997). Based on sequence homology and pharmacology, the metabotropic receptors are divided into three classes: Group I metabotropic receptors include mGlu1 and mGlu5; Group II metabotropic receptors include mGlu2 and mGlu3; and Group III metabotropic receptors include mGlu4, mGlu6, mGlu7 and mGlu8 (Conn and Pin, 1997). Investigations into the therapeutic potential of targeting metabotropic receptors have been hampered by the lack of systemically active and selective compounds to test in animal models of diseases. However, recently, a series of compounds including SIB-1757, SIB-1893, and 2-methyl-6-(phenylethynyl)-pyridine (MPEP), were described as being highly selective noncompetitive antagonists at the mGlu5 receptor (Varney et al., 1999; Gasparini et al., 1999). Subsequent studies, particularly with the systemically active antagonist MPEP, have allowed researchers to investigate the potential therapeutic effects of antagonizing mGlu5 receptors (Spooren et al., 2000; Tatarczynska et al., 2001).

Studies in whole animals using MPEP suggest that antagonists of mGlu5 receptors may be useful in the treat-

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ment of anxiety (Spooren et al., 2000; Tatarczynska et al., 2001). These published studies have examined the in vivo effects of MPEP in a variety of models of anxiety in both rats (social exploration, elevated plus maze, Geller–Seifter, fear-potentiated startle (FPS), and the conflict drinking test) and mice (stress-induced hyperthermia, marble burying, and the four-plate test) and reported qualitatively similar results to those seen with typical benzodiazepine anxiolytics (for review, see Spooren et al., 2001). However, a systematic comparison of the potency and efficacy of MPEP with a typical and an atypical anxiolytic in conditioned models of anxiety has not yet been reported. In order to evaluate the relative potency and efficacy as well as the potential use of mGlu5 receptor antagonists for the treatment of anxiety, we compared the effects of MPEP with two compounds used clinically to treat anxiety: buspirone (Rickels, 1987), a 5HT-1A partial agonist, and diazepam (Shader and Greenblatt, 1993), a GABA-A potentiator, in three models of conditioned anxiety in rats.

2. Methods

2.1. Animals

Naïve adult male Wistar rats (Charles River, 225–300 g) were used for FPS and ultrasonic vocalization (USV) studies. Animals were housed in groups of three under a 12-h light/dark cycle (lights on 06:30 h). The animals had free access to food and water. Twenty-five individually housed adult male Sprague–Dawley rats (Harlan, 290–330 g) were used for the Geller–Seifter test. These animals were fed daily 2 h after the completion of the session to maintain them at 85% of their free-feeding body weight. Animals had free access to water.

All studies were conducted in accordance with NIH guidelines for care and use of animals and were approved by the local IACUC.

2.2. Fear-potentiated startle

2.2.1. Training procedure

All animals were trained for 2 days prior to testing. Training consisted of placing the animals in a standard startle apparatus (SR-LAB, San Diego Instruments, San Diego, CA) where shock could be delivered from programmable electric shockers. On each of two consecutive days, the animals received 30 shocks (0.6 mA, 500 ms), each separated by 1 min. Each shock was preceded by the presentation of a 4-W light for 10 s. The chamber was dark between each presentation of the light and shock pairings.

2.2.2. Testing procedure

On the next day following training, animals were administered appropriate drug or vehicle and were placed in the startle apparatus for testing. Testing consisted of 42 presentations of an acoustic stimuli (95 dB, 20 ms) presented 30 s apart. According to a pseudorandom sequence, one half of the acoustic stimuli were preceded by 10 s of the presentation of the 4-W light. No shocks were administered on the test day. Data from the acoustic startle response were collected by force transducers located under the animals in the apparatus and expressed in constant arbitrary units (units were based on calibration with standard equipment). Data for each animal were separated into responses made in the presence of the light and those made in the dark (21 light, 21 dark) and expressed as the mean response for each animal.

2.3. Ultrasonic vocalization

2.3.1. Training procedure

All animals were trained for 2 days prior to testing. Training consisted of placing the animals in a standard operant chamber (ENV-018M, Med Associates, Georgia, VT) where shock could be delivered from a programmable shocker (Model ENV-413, Med Associates) and where it is equipped with an ultrasonic detector (Mini-3 Bat Detector, Ultra Sound Advice, UK). On each of two consecutive days, the animals received 20 shocks (1 mA, 4 s) separated by a random interval that averaged 60 s and ranging from 30 to 90 s. Each shock was administered concurrently with a 4-W light and an acoustic tone (85 dB, 4 s). The chamber was dark between each shock presentation.

2.3.2. Testing procedure

On the day following training, animals were administered appropriate drug/vehicle treatment and placed in the operant apparatus for testing. Testing consisted of 20 individual 4-s presentations of the acoustic tone and 4 W light presented according to a random interval that averaged 60 s (30–90 s). USVs (18–22 kHz) were recorded over the intertrial interval and were expressed as a sum of total time spent vocalizing for each animal. No shocks were administered on the test day.

2.4. Geller–Seifter

2.4.1. Training procedure

Naïve animals were food restricted to 85% of their free-feeding body weight and placed in a standard operant chamber (ENV-018M, Med Associates) equipped with a lever, house light, speaker, food dispenser, and a grid floor through which shock could be delivered from a programmable shocker. Training consisted of rewarding presses on the lever during house light illumination with food pellets (45 mg, BioServ, Frenchtown, NJ) over the course of a 30-min session. The number of lever presses required was gradually increased until animals were reliably pressing 30 times for one pellet delivery (FR-30). Once stable responding during the unpunished component had been established, a second component (punished) was introduced in which each FR-30 produced a food pellet accompanied by an

electric shock (0.2–0.8 mA for 500 ms). Punished and unpunished components were alternated during the session every 5 min with the punished component being signaled by an 80-dB tone. Shock levels were adjusted for each animal to produce at least a ratio of 5:1 in the rate of responding in the unpunished vs. punished components. Once stable responding had been established in the unpunished and punished components, the animals were placed on the testing schedule. The testing schedule was composed of three components: unpunished, punished, and time-out. During the time-out period, there was no light or tone and responses produced no programmed consequences. The three-component cycle was repeated twice per session.

2.4.2. Testing procedure

Testing began once stable rates of responding were observed over 5 days (no significant trend up or down). Overall, complete training typically took up to 4 months. Sessions were run 5 days per week with drugs (and corresponding vehicle treatments) given every Tuesday and Friday according to counterbalanced regimen. On rare occasions during nontreatment days (Monday, Wednesday, and Thursday), animals displayed abnormal rates of responding (i.e., >20% change from the animal's normal baseline) and were excluded from drug testing until normal responding returned for three consecutive sessions. Data were collected as rates of responding (responses per minute) from the unpunished and punished components and averaged over the entire session.

2.5. Drugs

Buspirone HCl was obtained from Sigma (St. Louis, MO), diazepam was obtained from Elkins-Sinn (Cherry Hill, NJ) and MPEP was generously provided by Merck chemistry department or purchased from Tocris (Bristol, UK). Buspirone was dissolved in physiological saline, diazepam was dissolved in 20% polyethylene glycol, and MPEP was dissolved in 10% Tween-80 (Sigma) and the pH was adjusted to ~pH 7 with several drops of NaOH. All drugs were administered in a volume of 1 ml/kg. Buspirone was administered intraperitoneally 30 min before testing, diazepam was administered subcutaneously 30 min before testing, and MPEP was administered intraperitoneally 1 h before testing. Doses were calculated as the total form.

2.6. Statistics

Data collected from FPS sessions were analyzed using a two-way repeated-measures ANOVA with Student–Newman–Keuls post hoc comparison procedure. ED_{50} values were calculated by taking the difference scores between the startle amplitude in the light minus the startle amplitude in the dark and interpolating a dose which reduced the difference to 50% of that observed in the vehicle control group. Data collected from USV sessions were analyzed with a (nonparametric) Kruskal–Wallis one-way ANOVA on ranks followed by Student–Newman–Keuls post hoc multiple comparison procedure. ED_{50} values were calculated by taking the median value of seconds vocalizing for the vehicle control group and interpolating a dose which reduced the median time spent vocalizing to 50% of that observed in the vehicle control group. Data collected from Geller–Seifter sessions were divided into punished and unpunished groups and were analyzed separately due to the nonnormal distribution of data from the punished component. Data from the unpunished component were

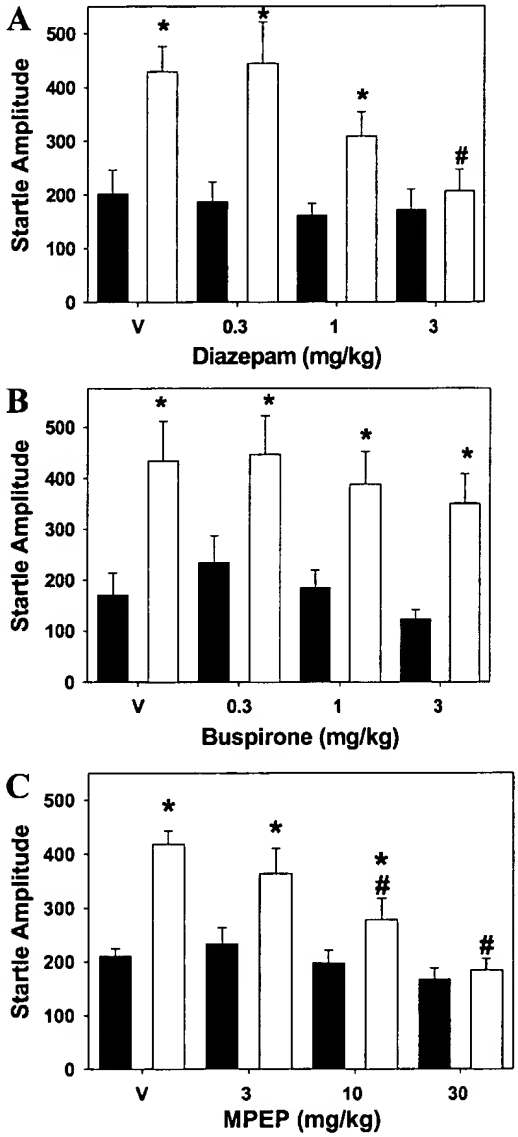

Fig. 1. The effect of diazepam (A), buspirone (B), and MPEP (C) on FPS. Closed bars represent the mean startle amplitude in the dark and open bars represent the mean startle amplitude in the presence of the shock-associated light cue. $n=8$ Wistar rats per bar set; $^{*}P<.05$ compared within dose group to startle in the dark; $^{\#}P<.05$ compared to light cue startle in the vehicle control group.

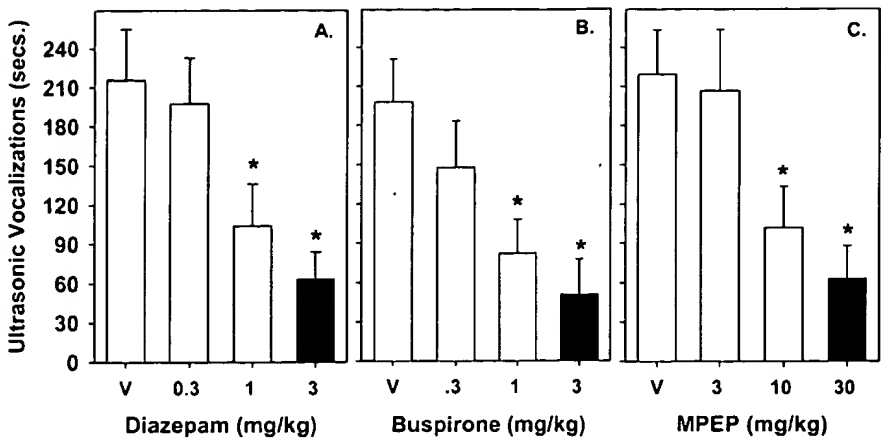

Fig. 2. The effect of diazepam (A), buspirone (B), and MPEP (C) on conditioned USVs. Grey-scale bars represent the mean total seconds of USVs recorded over the entire session. $n = 18$ Wistar rats per bar; $^*P < .05$ compared to the vehicle control group.

analyzed using a one-way repeated-measures ANOVA followed by Student–Newman–Keuls post hoc multiple comparison procedure. Data from the punished component were analyzed using a Friedman repeated-measures ANOVA on ranks followed by Student–Newman–Keuls post hoc multiple comparison procedure. For all statistical comparisons, a $P < .05$ was used for determining statistical significance.

3. Results

The effect of diazepam, buspirone, and MPEP on FPS is shown in Fig. 1A–C. In the FPS test, anxiety is indicated when the startle response in the light is greater than the startle response in the dark. A significant difference between the response in the vehicle group and the drug-treated group in the light suggests that the dose has produced an anxiolytic effect. Multivariate analysis using a two-way ANOVA and post hoc SNK test determined that 3 mg/kg diazepam significantly decreased startle amplitude in the light vs. vehicle ($P < .05$). Also, all treatment groups except 3 mg/kg diazepam displayed a significant enhancement of startle amplitude in the presence of the light vs. startle amplitude in the dark ($P < .05$). In contrast to diazepam, buspirone was not active in the FPS model (Fig. 1B), since it did not significantly decrease startle amplitude in the light vs. the vehicle group ($P > .05$). Also, all treatment groups displayed a significant enhancement of startle amplitude in the presence of the light vs. startle amplitude in the dark ($P < .05$).

The effect of the mGlu5 antagonist, MPEP, on FPS is shown in Fig. 1C. Doses of 10 and 30 mg/kg MPEP significantly decreased startle amplitude in the light vs. vehicle ($P < .05$). Also, all treatment groups except 30 mg/kg MPEP displayed a significant enhancement of startle amplitude in the presence of the light vs. startle amplitude in the dark ($P < .05$). Comparing the potency of diazepam and MPEP, diazepam was approximately four times as potent as MPEP at decreasing potentiation of startle ($ED_{50} = 1.4$ mg/kg diazepam vs. 5.6 mg/kg MPEP), although both compounds showed complete reversal of the startle amplitude.

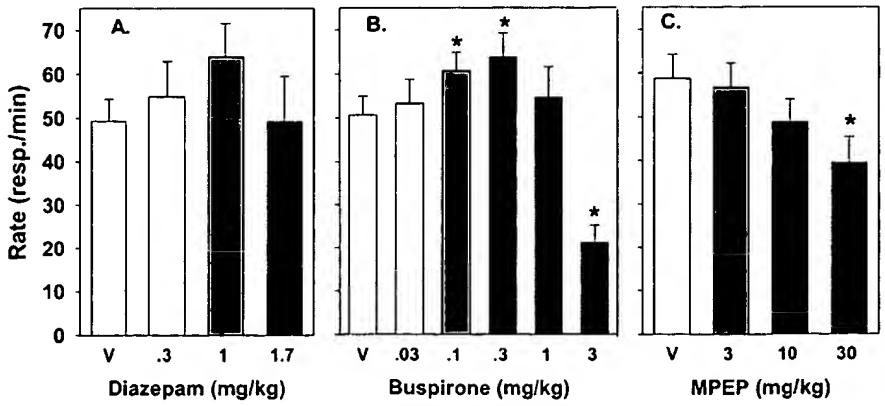

Fig. 3. The effect of diazepam (A), buspirone (B), and MPEP (C) on *unpunished* responding in the Geller–Seifter assay. Grey-scale bars represent the mean rate of responding recorded over the entire session. $n = 15–16$ Sprague–Dawley rats per dose–effect curve; $^*P < .05$ compared to within-subject vehicle control.

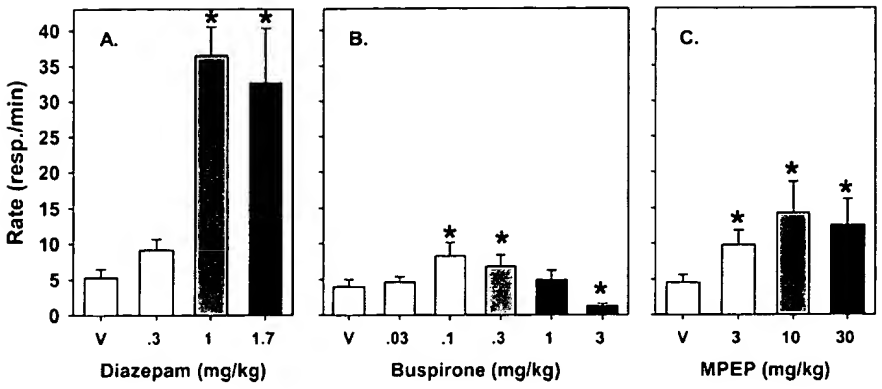

Fig. 4. The effect of diazepam (A), buspirone (B), and MPEP (C) on *punished* responding in the Geller–Seifter assay. Grey-scale bars represent the mean rate of responding recorded over the entire session. $n = 15–16$ Sprague–Dawley rats per dose–effect curve; * $P < .05$ compared to within-subject vehicle control.

The effect of diazepam on conditioned USVs is shown in Fig. 2A. In this test, anxiety is indicated when the animal exhibits high amounts of vocalizations in the ultrasonic range. A significant comparison between the responses in the vehicle and drug-treated group suggests that the drug produced a significant anxiolytic-like effect. Diazepam (at 1 and 3 mg/kg), buspirone (at 1 and 3 mg/kg), and MPEP (at 10 and 30 mg/kg) significantly decreased conditioned USVs ($P < .05$, Fig. 2A, B, and C, respectively). All three compounds decreased USVs to a similar degree, although MPEP was less potent ($ED_{50} = 6$ mg/kg) than diazepam or buspirone ($ED_{50} = 0.5$ and 0.3 mg/kg for diazepam and buspirone, respectively).

In the Geller–Seifter assay, anxiety is indicated when the rate of responding during the punished component of the test is lower than the rate of responding in the unpunished component of the test (Pollard and Howard, 1990). A treatment that produces a significant increase in the rate of responding in the punished component relative to that observed upon vehicle administration suggests that the treatment has produced a significant anxiolytic-like effect. The effect of diazepam on unpunished rates of responding in the Geller–Seifter assay is shown in Fig. 3A. Doses of diazepam of 1.7 mg/kg, or below, did not decrease unpunished responding. However, a dose of 3 mg/kg diazepam was not tested as early dose-ranging studies suggested that this produced a near complete suppression of responding in both components of the test, and maximal unpunished rates of responding were observed at doses lower than the highest dose tested (1.7 mg/kg).

The effect of buspirone on unpunished rates of responding in the Geller–Seifter assay is shown in Fig. 3B. The highest dose of buspirone tested, 3 mg/kg, significantly ($P < .001$) decreased the unpunished rate of responding. Likewise, the highest dose of MPEP that was tested, 30 mg/kg, also significantly ($P < .001$) decreased the unpunished rate of responding in the Geller–Seifter assay (Fig. 3C).

The effect of diazepam on punished rates of responding is shown in Fig. 4A. Doses of 1 and 1.7 mg/kg diazepam significantly increased the punished rate of responding ($P < .05$). The effect of buspirone on punished rates of responding is shown in Fig. 4B. At doses of 0.1 and 0.3 mg/kg, buspirone significantly increased the punished rate of responding ($P < .05$), while the higher dose of 3 mg/kg buspirone decreased the punished rate of responding ($P < .05$). The effect of MPEP on punished rates of responding is shown in Fig. 4C. All doses (3, 10, and 30 mg/kg) of MPEP significantly increased the punished rate of responding ($P < .05$). The increase in punished responding rate observed with diazepam was notably larger than rates observed with either buspirone or MPEP (Fig. 4).

4. Discussion

Recent data suggest that the mGluR5 antagonist, MPEP, demonstrates anxiolytic activity in a number of animal models of anxiety. For example, Spooren et al. (2000) and Schulz et al. (2001) examined the effects of MPEP in a number of assays of conditioned and unconditioned anxiety. The present report confirms the anxiolytic-like activity of MPEP in the conditioned anxiety assays of the Geller–Seifter and FPS assays and extends those findings with anxiolytic-like activity in an assay of conditioned USVs. When compared to the typical anxiolytic diazepam, MPEP showed a qualitatively similar pattern of effects with activity in all three assays. Buspirone, on the other hand, only showed activity in two of the three assays (conditioned USVs and the Geller–Seifter). Taken together, these results suggest that MPEP may possess a range of anxiolytic-like activity greater than buspirone; similar to the range of activity seen with benzodiazepines like diazepam.

The FPS procedure has been used extensively to assess potential anxiolytic effects of compounds in rats (for review, see Davis et al., 1993). Briefly, this assay assesses anxiety by

eliciting an acoustic startle response both in the presence and in the absence of a cue that has been classically conditioned to be associated with a brief aversive shock. When the animal is expecting the aversive shock (in the presence of the associated cue), the acoustic startle response is greater than when the animal is responding in the absence of the associated cue and this enhancement of startle response is used as an index of anxiety. Compounds that decrease anxiety in humans, like benzodiazepines, have been shown to decrease FPS (e.g., Davis, 1979). Also, the atypical anxiolytic buspirone, which has a more limited spectrum of anxiolytic activity in humans (Sheehan et al., 1990), has been shown to decrease FPS by others (Mansbach and Geyer 1988; Kehne et al., 1988). In the present study, both diazepam and MPEP decreased FPS, whereas buspirone did not show anxiolytic-like activity in this assay. The activity of MPEP in this assay is consistent with the recent report by Shulz et al. (2001) showing a maximal effect of MPEP at 30 mg/kg. While buspirone did not show anxiolytic-like activity in this study, in previous studies, we have observed anxiolytic-like activity of buspirone in the FPS assay when using the Long–Evans strain of rats rather than Wistar rats (data not shown). Additionally, our laboratory regularly uses more conditioning than those studies that have reported positive effects of buspirone (60 pairings of conditioned and unconditioned shock pairing vs. 20 pairings). Taken together, the differences in strains and conditioning paradigms or some interaction thereof may account for the apparent discrepancy between the present results using buspirone and those of other investigators. It is our feeling that this discrepancy suggests that the conditions used in the current report may be eliciting a higher degree of anxiety than those previously reported and, as such, the assay conditions may be more conservative in assessing anxiolytic-like activity than those commonly reported in the literature. Diazepam and MPEP showed similar efficacy at the highest dose tested in this assay in that both compounds could produce a complete reversal of potentiation of startle (i.e., no statistically significant difference between startle response in the light vs. the startle response in the dark).

Rats emit USVs when placed in situations that might reasonably be considered to elicit a heightened state of anxiety. Published examples of these phenomena include the recording of USVs upon the withdrawal from habit-forming drugs such as cocaine (e.g., Barros and Miczek, 1996) or ethanol (Knapp et al., 1998). USVs can also be evoked with aversive stimuli such as air puffs or shocks (De Vry et al., 1993; Knapp and Pohorecky, 1995), in response to agonistic encounters with other rats (Vivian and Miczek, 1993), and following classically conditioned anxiety (Molewijk et al., 1995). Furthermore, these USVs are sensitive to both typical and atypical anxiolytics (Molewijk et al., 1995; De Vry et al., 1993; Vivian and Miczek, 1993). Consistent with this literature, the present report found that both diazepam and buspirone reliably reduced USVs in a classically conditioned model of anxiety. Similar to these reference anxiolytics, MPEP also decreased USVs in this assay. All three compounds showed similar efficacy at the highest doses tested (70–75% inhibition of USVs).

The Geller–Seifter assay of punished responding has been used extensively for the investigation of potential anxiolytic effects of compounds in animals (e.g., Riblet et al., 1982; Spooren et al., 2000). In this assay, operant responding reinforced with food is alternated with responding that is both reinforced with food and punished with an electric shock. Benzodiazepines and barbiturates reliably increase rates of punished responding and show good anxiolytic activity in humans. Buspirone has been reported to produce a range of effects from no effect on punished responding (Sanger, 1990) to modest increases in punished responding (Riblet et al., 1982; Weissman et al., 1984; Young et al., 1987). The current findings are generally consistent with those reported in the literature with diazepam producing a 6.6-fold and buspirone producing a 2-fold increase in punished responding. MPEP produced an intermediate (3-fold) increase in punished responding. While Spooren et al. (2000) reported that MPEP did not produce statistically significant increases in punished responding in the Geller–Seifter assay, a trend toward increasing rates of punished responding was observed. One possible explanation for the different results from these two studies is that we employed slightly different assay parameters. Spooren et al. used a variable-time 10 s (VI-10) while we used an FR-30 that resulted in less inhibition of responding during the punished component (<1 vs. 4–6 responses/min) under vehicle-treated conditions. Thus, the current assay conditions may have required less disinhibition to produce statistically significant results. While all three compounds produced significant increases in punished responding, diazepam had the largest effect followed by MPEP then buspirone.

Compounds that affect motor coordination or produce sedation will confound results from behavioral studies, including the anxiety models in this study. Spooren et al. (2000) and Shulz et al. (2001) addressed potential side effects of MPEP by looking at spontaneous locomotor activity and reported no significant effects up to 100 and 30 mg/kg, respectively. However, we found that MPEP produced nonselective effects on behavior at a dose of 30 mg/kg (ip), producing a statistically significant decrease in the rate of responding in the unpunished component of the Geller–Seifter assay. While the previous reports used locomotor activity to address potential side effects, drug effects on operant responding are usually similar and are often interpreted in terms of potential side effects as well. In the previous two studies, MPEP was administered per os whereas the present study used intraperitoneal administration, so direct dosage comparisons are difficult. Using the minimum dose that produced a statistically significant decrease in unpunished responding reported in the current study as a measure of potential side effects, we are able to make some estimates as to the behavioral selectivity of the compounds for anxiolytic-like activity. Within the Geller–

Seifter assay, buspirone exhibited a 30-fold window between anxiolytic-like activity and potential side effects followed by MPEP at 10-fold and diazepam at 3-fold. All three drugs exhibited a 3-fold window using effects on USVs as the anxiolytic-like activity measure. Furthermore, in the FPS, MPEP exhibited a 3-fold window while diazepam exhibited no window at all with a dose ratio of 1. Averaging across all three assays, MPEP displayed a 5-fold anxiolytic-like behavioral selectivity while diazepam's selectivity was only 2-fold. Despite the discrepancy with previous reports that suggested MPEP produces no side effects, the current study is consistent with the previous conclusions that MPEP may display a larger therapeutic window than typical anxiolytics. The side effects produced by MPEP may be attributed to weak antagonisms of NMDA receptors at high doses (O'Leary et al., 2000).

The mechanisms through which blockade of mGlu5 results in anxiolytic-like behaviors in rats are unknown. Likely structures involved in these models include the hippocampus and amygdala. Both regions show abundant expression of mGlu5 receptors (Fotuhi et al., 1994; Romano et al., 1996). The ability of a nonselective Group I mGlu antagonist to produce anxiolytic-like responses in the Vogel test were observed following intrahippocampal administration (Chojnacka-Wojcik et al., 1997), further suggesting that this structure might be related to anxiolytic effects. To verify these hypotheses, experiments with brain region-specific injections of MPEP are in progress.

Overall, the results reported in this study confirm and extend the literature reports suggesting that MPEP produces anxiolytic-like activity in animal models. Furthermore, the pattern of results suggests that MPEP may have greater efficacy than buspirone and a larger therapeutic index than diazepam. Studies examining the abuse potential of and tolerance to MPEP would be valuable in determining whether mGlu5 receptor antagonists may provide a new therapeutic approach for treating anxiety disorders in humans.

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Anxiolytic-Like Effects of the Prototypical Metabotropic Glutamate Receptor 5 Antagonist 2-Methyl-6-(phenylethynyl)pyridine in Rodents

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ABSTRACT

Recently, selective and systemically active antagonists for the metabotropic glutamate 5 receptor ($mGlu_5$) were discovered, and the most potent derivative was found to be MPEP (2-methyl-6-(phenylethynyl)pyridine). Given the high expression of $mGlu_5$ receptors in limbic forebrain regions, it was decided to evaluate the anxiolytic potential of MPEP. After an acute oral administration, MPEP attenuated the anxiety-dependent variable in a variety of well established anxiety test paradigms. In rats, MPEP (10, 30, and 100 mg/kg) increased punished responses in the Geller-Seifter test, but none of these effects reached statistical significance. MPEP significantly increased the ratio (open/total arm entries; 0.1, 1, and 10 mg/kg), the number of open arm entries (0.1, 1, and 10 mg/kg), as well as time spent on open arm (0.1 and 1 mg/kg) in the elevated plus maze test. Furthermore, MPEP (0.3 and 1 mg/kg) significantly increased the time spent in social contact in the social exploration test. In mice, MPEP attenuated stress-induced hyperthermia (15 and 30 mg/kg) and decreased the number of buried marbles in the marble burying test (7.5 and 30 mg/kg). Finally, MPEP (0.01, 0.1, 1, 10, and 100 mg/kg) was tested on spontaneous locomotor activity in mice, and only a dose of 100 mg/kg significantly reduced vertical activity; no effect was seen on horizontal activity. MPEP (7.5, 15, and 30 mg/kg) was ineffective on *d*-amphetamine-induced (2.5 mg/kg) locomotor activity in mice and prepulse inhibition in rats (1, 3, or 10 mg/kg). Thus, these findings indicate that MPEP exhibits anxiolytic-like effects and low risks for sedation and psychotomimetic side-effects in rodents.

It is widely accepted that glutamate is the main excitatory neurotransmitter in the brain (McGeer et al., 1987). Glutamate mediates its effect via two distinct types of receptors, i.e., the ionotropic receptors and the metabotropic receptors (Monaghan et al., 1989; Conn and Pin, 1997). The family of the metabotropic receptors (mGlu) contains of, at present, eight different subtypes (Conn and Pin, 1997). On the basis of sequence homology, effector coupling, and pharmacology, mGlu receptors are divided into three subgroups. The group I mGlu receptors ($mGlu_1$ and $mGlu_5$) are positively coupled to phospholipase C, and the group II mGlu receptors ($mGlu_2$ and $mGlu_3$) and the group III receptors ($mGlu_4$, $mGlu_6$, $mGlu_7$, and $mGlu_8$) are negatively coupled to adenylate cyclase (Pin and Duvoisin, 1995; Conn and Pin, 1997).

Drugs targeting ionotropic receptors have so far failed to qualify as therapeutics, not because of lack of efficacy but mainly due to the induction of severe and persistent side-effects, i.e., most prominently psychotomimetic effects (Danysz et al., 1996). Currently, agonists or antagonists of metabotropic glutamate receptors are believed to have a milder side effect profile and, accordingly, compounds specifically interacting at these receptors have been proposed as potential new therapeutics for a number of neurological and psychiatric disorders (Knöpfel et al., 1995; Conn and Pin, 1997; Nicoletti et al., 1997). However, these hypotheses originate from speculations based on the expression pattern of distinct mGlu-subtype receptors in the central nervous system and on the effects of nonselective compounds, which do not discriminate between distinct mGlu-receptor subtypes.

After the discovery of selective and systemically active antagonists for the $mGlu_5$ receptor, it is now possible to study the potential role of this receptor subtype in behavior and disease models (Gasparini et al., 1999). In cells expressing the human $mGlu_5$ receptor, the most potent derivative, 2-methyl-6-(phenylethynyl)pyridine (MPEP), completely inhibited quisqualate-stimulated phosphoinositide hydrolysis

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ABBREVIATIONS: mGlu, metabotropic glutamate receptor; MPEP, 2-methyl-6-(phenylethynyl)pyridine; SIH, stress-induced hyperthermia; PPP, prepulse pulse; PA, pulse alone; PPI, prepulse inhibition; (+)-MK801, (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine hydrogen maleate.

with an IC_{50} value of 36 nM. When tested at group II and III receptors, MPEP did not show agonist or antagonist activity at 100 μM on human $mGlu_2$, $mGlu_3$, $mGlu_{4a}$, $mGlu_{7b}$, and $mGlu_{8a}$ receptors nor at 10 μM on the human $mGlu_6$ receptor. Electrophysiological recordings in *Xenopus laevis* oocytes demonstrated no significant effect at 100 μM on human NMDA (NMDA1A/2A), rat AMPA [Glu3-(flop)], and human kainate [Glu6-(IYQ)] receptor subtypes nor at 10 μM on the human NMDA1A/2B receptor (Gasparini et al., 1999). MPEP was also tested in a binding battery of receptors containing representatives of monoamine receptor subtypes (adrenaline, dopamine, serotonine), muscarinic, nicotinic, neurokinin, GABA-A, GABA-B, and adenosine receptors. MPEP did not show significant binding affinity for any of the receptors tested up to a concentration of 10 μM (F. Gasparini, manuscript in preparation). Furthermore, when tested for oral bioavailability and blood-brain barrier penetration, MPEP was found to be well absorbed and to readily penetrate the brain 1 h after administration (F. Gasparini, manuscript in preparation).

$mGlu_5$ receptors are widely expressed in the central nervous system with a particularly high expression in the hippocampus, the nucleus accumbens, and the striatum but also in the internal and external pallidal segments and the substantia nigra pars reticulata (Shigemoto et al., 1993; Testa et al., 1994; Romano et al., 1995). These brain areas are well known to represent key elements in the so-called corticobasal ganglia-cortico circuitry (Albin et al., 1989; Chesselet and Delfs, 1996), i.e., circuits involved in emotional processes such as anxiety (Duncan et al., 1996). Thus, given the high expression of $mGlu_5$ receptors in limbic forebrain regions, it was decided to evaluate the potential of MPEP in a multiplicity of well established animal models of anxiety as recently reviewed by Olivier et al. (2000) and Rodgers (1997), that included a variety of nonconditioned and conditioned anxiety models with a wide range of different behaviors and motivations. In addition, the effect of MPEP on locomotion was studied to obtain an index of the specificity of anxiolytic action as well as its effect on *d*-amphetamine-induced locomotor activity to explore the mechanism of action of MPEP-mediated effects. Finally, to investigate also the potential for psychotomimetic side-effects, MPEP was tested on prepulse inhibition (PPI).

Materials and Methods

Social Exploration

Animals. Adult male Sprague-Dawley rats (="resident" rats; OFA/IC, Iffa Crédo, Les Oncins, France; 350–400 g) and young Lister Hooded rats (="intruder" rats; LI/HO, Harlan, Horst, The Netherlands; 100–120 g) were used. Intruder rats were housed in pairs and resident rats were individually housed in macrolon cages (42 × 26 × 15 cm) for 2 weeks before the test. All animals were housed in the same room. The housing facility was temperature- and humidity-controlled and equipped with artificial illumination (6:00 AM to 6:00 PM, lights on). The animals had access to water and food (Ecosan, Eberle Nafag AG, Gossau, Switzerland), ad libitum. All rats were experimentally naïve.

Drug Treatment and Experimental Procedure. Animals received MPEP [doses: 0.003, 0.3, or 1 mg/kg (experiment 1) or 1 or 10 mg/kg (experiment 2); the results of the first experiment suggested a bell-shaped dose-response effect and the second experiment was used to further explore these findings], chlordiazepoxide-HCl (5 mg/kg, p.o.; CDZ, Research Biochemicals International, Natick, MA), i.e., the reference compound, or vehicle (0.5% methylcellulose; Animed). The injection volume was 2 ml/kg. Oral treatment was given to the intruder rat only, and the test was performed 1 h after drug administration. All observations were made during the light phase (8:00 AM to 1:00 PM) in the home cage of the resident rat (see above). The floor of the cage was covered with sawdust. Pairs consisting of one intruder rat and one resident rat were assigned at random to one of the experimental or the control groups. The duration of active approach behaviors (=time spent in social activity) of the intruder rat (sniffing, anogenital exploration, nosing, grooming, licking, playing) toward the resident was manually scored and cumulatively recorded over a period of 5 min.

Statistics. The statistical evaluation was performed on pooled data of two independent experiments [dependent variable: time spent in social contact (see above)] using a one way ANOVA followed by Dunnett's test for comparison of multiple dose levels against vehicle (SigmaStat 2.03; SPSS, Chicago, IL).

Elevated Plus Maze

Animals. Male adult Sprague-Dawley rats (Iffa Crédo, Les Oncins, France; 180–220 g) were housed in groups of four in macrolon cages (42 × 26 × 15 cm) for at least 3 days before the experiment. The housing facility was temperature- and humidity-controlled and equipped with artificial illumination (6:00 AM to 6:00 PM, lights on). The animals had access to water and food (Ecosan, Eberle Nafag AG), ad libitum. All animals were experimentally naïve.

Apparatus. The elevated plus-maze consists of two open arms (40 × 12 cm) and two enclosed arms (40 × 12 × 20 cm), which all extend from a common central platform (12 × 12 cm). The configuration forms the shape of a plus sign, with similar arms arranged opposite to each another, and the apparatus is elevated 60 cm above the floor on a central pedestal. The maze is made from gray Plexiglas. The grip on the open arms is facilitated by inclusion of a small raised edge (0.25 cm) around their perimeter.

Drug Treatment and Experimental Procedure. The method was adopted from Handley and Mithani (1984). Rats were randomly allocated to one of the various treatments. Animals were transported from the housing room to the laboratory at least 1 h before testing. After oral drug administration, rats were individually housed in macrolon cages (22 × 16 × 14 cm), and after 60 min placed onto the central platform facing an enclosed arm. An 8-min trial was performed, and the maze was thoroughly cleaned between subjects. Direct registrations were made by an observer sitting close to the maze, and the following conventional parameters were used: number of open and closed arm entries (arm entry defined as all four paws entering an arm) and time spent on open arms (excluding the central platform). Animals from the different treatment groups were alternatively tested, and trials were performed between 8:30 AM and 12:30 PM, i.e., within the first half of the light phase.

Rats were treated with MPEP [doses: 0.1, 1, or 10 mg/kg, p.o. (n = 15 per group)], chlordiazepoxide-HCl (10 mg/kg, p.o.; Research Biochemicals International), i.e., the positive control, or vehicle (0.5% methylcellulose; Animed).

Statistics. For each behavioral parameter a separate ANOVA was performed followed by Dunnett's multiple comparison test to compare different dose levels against vehicle (SYSTAT 8.0; SPSS).

Stress-Induced Hyperthermia and Marble Burying

Animals. Male mice (OF1/IC; Iffa Crédo, Les Oncins, France; 18–20 g) were housed in macrolon cages (42 × 26 × 15 cm; n = 15 per cage) in the laboratory in which the animals were later tested. The room was temperature-controlled and equipped with artificial illumination (6:00 AM to 6:00 PM, lights on). The animals had free access to water and food (Ecosan, Eberle Nafag AG), ad libitum. All mice were experimentally naïve.

Stress-Induced Hyperthermia. The test procedure for stress-induced hyperthermia (SIH) was adopted with minor modification from the original description by Lecci et al. (1990). Briefly, rectal temperature was measured to the nearest 0.1°C by a thermometer (ELLAB instruments, Copenhagen, Denmark) via a lubricated thermistor probe (2-mm diameter) inserted 20 mm into the rectum while the mouse was hand-held near the base of the tail. The probe was left in place until steady readings were obtained (within 15 s).

Drug Treatment and Experimental Procedures. Fifteen animals were housed per macrolon cage (42 × 26 × 15 cm). At least 24 h before the experiment animals within a cage were marked on their fur with color for later identification. Sixty minutes before taking the rectal temperature all individuals within a given cage were consecutively treated at 1-min intervals with MPEP (doses: 1.5, 7.5, 15, or 30 mg/kg, p.o.; injection volume: 10 ml/kg), chlordiazepoxide-HCl (10 mg/kg, p.o.; Research Biochemicals International), i.e., the positive control, or vehicle (0.5% methylcellulose; Animed). Exactly 60 min later the mice were consecutively removed from the cage (again at 1-min intervals), and rectal temperature was determined and noted. Once temperature had been recorded, the animals were placed in a different (adjacent) cage. The dependent variable, i.e., the stress-induced hyperthermia, was defined as the delta of the median rectal temperature within the six initially removed mice and the median rectal temperature within the six last removed mice within a cage. This delta was calculated for six to eight cages depending on the specific treatment group (see Fig. 3 legend), whereas in the final representation the mean of these six to eight values was used. The rectal temperature of the very first animal was used, in addition, to evaluate the compound's potential effect on basal body temperature, per se.

Marble Burying. The test procedure for marble burying was adopted with minor modifications from the original description of Broekkamp et al. (1986). Briefly, the first two mice removed from the cage while assessing stress-induced hyperthermia were used in the marble burying test. The animals were individually placed in small cages (22 × 16 × 14 cm) in which 10 marbles had been equally distributed on top of a 5-cm sawdust bedding. The mice were left undisturbed in these cages for 60 min; after removal of the mouse the number of visible, nonburied marbles (i.e., less than two-thirds covered by sawdust) was counted and this number served as the dependent variable.

Statistics. Stress-induced hyperthermia (delta of rectal temperature) and marble burying (number of visible marbles) were statistically evaluated using a Kruskal-Wallis one-way ANOVA followed by a post hoc one-tailed Mann-Whitney *U* test, Bonferroni corrected (SYSTAT 8.0).

Geller-Seifter Conflict Test in Rats

Animals. Male Wistar rats (Elevage Janvier, Le Genest-Saint-Isle, France; 180–240 g) were housed in macrolon cages (41 × 25 × 14 cm; $n = 5$ per cage). The animal room was temperature-controlled and equipped with artificial illumination (6:00 AM to 6:00 PM, lights on). The animals had access to water and food (UAR, Villemoisson-sur-Orge, France), ad libitum. All rats were experimentally naïve.

Drug Treatment and Experimental Procedures. The method applied here was adopted from Geller and Seifter (1960) and included the modification put forward by Davidson and Cook (1969). Animals were trained in sound-attenuated standard Skinner boxes (23 × 21 × 18 cm; MED Associates, St. Albans, VT), which were fitted with a white house light, a red signal light, a lever (force necessary to depress lever: 25 g), and a food pellet dispenser. The lever was positioned on the right side of the food receptacle, which was itself connected to the pellet dispenser. The Skinner boxes were connected to a MED-PC programming system that controlled the experiment and automatically collected the data.

Training Procedure. Rats were submitted to daily training sessions (15 min) according to a variable interval, 15-s reinforcement schedule. In this schedule, only those responses occurring after variable intervals (mean value: 15 s) were rewarded. These reinforced responses consisted of the delivery of a 45-mg food pellet (Noyes, Lancaster, UK). The rats were then submitted to three nonpunished periods of 3 min each, signaled by the presence of the white house light, alternated with two punished periods of 3 min each, signaled by the presence of a red signal light, during which lever pressing was simultaneously reinforced and punished with electric foot-shock according to a variable ratio reinforced schedule (punished periods). Reinforcement and shocks were given after a variable number of responses (mean value: 10) and foot-shocks (0.4 mA, 0.5 s) were delivered by a scrambled shock generator (model E1308; Coulbourn Instruments, San Diego, CA). Daily sessions lasted 15 min. The animals received a p.o. administration of distilled water 60 min before each session. In addition to the food pellets consumed in the Skinner box, animals received a 15-g food ration in their home cages. This amount of food was given after the last animal was tested and represented around 80% of the unlimited daily food intake.

Three dependent variables were used: 1) The number of punished responses—the total number of presses on the lever during the punished periods; 2) The number of shocks—the total number of shocks the animal received during the punished periods; and 3) The number of nonpunished responses—the total number of lever-presses during the nonpunished periods.

Drug Testing Procedure. Drug testing was started once the rats showed stable baseline performance and had demonstrated a positive response to the reference anxiolytic chlordiazepoxide-HCl (16 mg/kg, p.o.; CDZ, Research Biochemicals International). Sessions with MPEP (doses: 10, 30, or 100 mg/kg, p.o.) were run twice weekly with at least one drug-free training session (oral treatment with distilled water) in between. During the training phase, drug sessions lasted 15 min and the food regime was similar to the training period. Each animal was used as its own control and received all treatments in a randomized order to ensure even distribution of the different treatments in time. Test drug or vehicle (0.5% methylcellulose) was administered 60 min before the test. Each of the eight rats was always tested in the same Skinner box and at the same time of day.

Statistics. Data were analyzed using a paired Student's *t* test, which was Bonferroni-corrected.

Spontaneous Locomotor Activity Test

Animals. Male OF1/IC mice (Iffa Crédo, Les Oncins, France; 18–20 g) were housed in macrolon cages (42 × 26 × 15 cm, $n = 10$ per cage) in a temperature-controlled room under artificial illumination (6:00 AM to 6:00 PM, lights on) and had access to water and food (Ecosan, Eberle Nafag AG), ad libitum.

Drug Treatment and Experimental Procedures. Mice received an oral injection of MPEP (doses: 0.01, 0.1, 1, 10, or 100 mg/kg, p.o. (experiment 1), or 7.5, 15, or 30 mg/kg, p.o. (experiment 2)) or vehicle (0.5% methylcellulose; Animed). Subsequently, the animals were individually placed into locomotor activity cages (17 × 32 × 20 cm; Motron motility, Novartis AG), and the number of beam interruptions at two different heights (2.5 and 11 cm) was registered for 120 min and used to quantify horizontal and vertical activity, respectively.

Statistics. A separate one-way ANOVA was used to evaluate total horizontal or vertical activity counts in a 120-min period of registration (SYSTAT 8.0).

d-Amphetamine-Induced Locomotor Activity

Animals. Male OF1/IC mice (Iffa Crédo, Les Oncins, France; 18–20 g) were housed in macrolon cages (42 × 26 × 15 cm, $n = 10$ per cage) in a temperature-controlled room under artificial illumination (6:00 AM to 6:00 PM, lights on) and had access to water and food (Ecosan, Eberle Nafag AG), ad libitum.

Drug Treatment and Experimental Procedures. Horizontal locomotor activity was assessed in transparent Plexiglas boxes (dimensions: 19 × 31 × 16 cm), and activity was detected and registered

using the TSE Moti system (TSE, Bad Homburg, Germany), which is based on the registration of infrared light beam interruptions along the x, y, and z axes, as caused by an animal's movements; data were directly stored in a computer. Mice were individually placed in the Plexiglas boxes and allowed to habituate for 45 min. Then the animals were removed from the boxes and injected with MPEP (7.5, 15, or 30 mg/kg, p.o.) or its solvent (methylcellulose, 0.5%) and then immediately returned to their respective boxes. Fifteen minutes later the animals were again removed from the boxes and injected with d-amphetamine (2.5 mg/kg, i.p.) or its solvent (distilled water). The animals were again immediately returned to their respective locomotor boxes, and the horizontal locomotor activity was registered for the next 120 min. The dose of d-amphetamine was chosen to allow either inhibition or potentiation to be seen.

Statistics. A separate two-way ANOVA (factors: MPEP and d-amphetamine) was used to evaluate total horizontal or vertical activity counts during 120 min of registration (SYSTAT 8.0).

Prepulse Inhibition

Animals. Male adult Brown Norway rats (Iffa Crédo, L'Arbresle, France; 214–245 g) were housed in groups of four in macrolon cages (42 × 26 × 15 cm) for at least 3 days before the experiment. The housing facility was temperature- and humidity-controlled and equipped with artificial illumination (6:00 AM to 6:00 PM, lights on). The animals had access to water and food (Ecosan, Eberle Nafag AG), ad libitum. All animals were experimentally naïve.

Apparatus. PPI was measured with a commercially available Coulbourn startle system (Coulbourn Instruments), modified such that all acoustic stimuli were presented to the animals via a single Visaton (Germany) wide range tweeter (type DHT 9 AW-NG) in the center of the ventilated, sound-attenuated test chamber. White noise was used for background, prepulse, and startle pulse stimuli with a frequency range of the tweeter around 4 kHz. Sound pressure levels were calibrated on the db-A scale using a Bruel and Kjaer (Copenhagen, Denmark) 4133 microphone and 2209 type meter (Naerum, Denmark). The startle response was recorded with a quartz force sensor for measuring dynamic and quasistatic forces (Kistler Instruments AG, Winterthur, Switzerland; type 9203; connected to a Kistler charge amplifier type 5011, with low pass filter at 300 Hz and high pass at 100 s). The sensor was mounted directly below the animal enclosure (plastic box covered with metal grid; 16 × 8 × 8 cm) and calibrated using weights in the range between 10 and 1500 g. The output signal of the charge amplifier was digitized (sample rate, 1 kHz for 200 ms, 8-bit) and stored on a microcomputer.

Drug Treatment and Experimental Procedures. Animals were pretreated with MPEP (1, 3, or 10 mg/kg, p.o.) or vehicle (0.5% methylcellulose, 2 ml/kg). Alternatively, animals were injected with (+)-MK801 (0.1 mg/kg, s.c.) or saline (1 ml/kg). 30 min after the administration of (+)-MK801, or 60 min after MPEP treatment, animals were positioned in the startle test chamber, such that at least one subject from each treatment group was included in each session. From session to session, the different treatment groups were assigned to different startle sensors (clockwise rotation). This procedure was used to rule out artifacts related to sensor and/or session differences. Background noise was continuous at a level of 62 db. Acoustic stimuli consisted of a startle-eliciting stimulus of 105 db for 40 ms and prepulses of 4, 8, or 16 db above background with a duration of 20 ms. The startle-eliciting stimulus was presented either alone (pulse alone, PA) or in combination with a prepulse presented 100 ms earlier (prepulse pulse, PPP). A startle session included an adaptation time of 3 min and subsequently of 63 stimuli. The first three stimuli were PA stimuli that were not included in the analysis; these merely served to achieve a stable baseline in startle reactivity. Subsequently, three blocks of 10 PA stimuli were presented (PA1, PA2, and PA3, respectively). The second block included in addition 30 PPP stimuli (10 of each type), whereby stimuli in this block were presented in randomized order. The interval between stimuli was randomized between 9 and 21 s. Startle peak amplitudes (g) were estimated for each animal averaged over the 10 stimuli of one type. Prepulse inhibition was computed according the formula, %PPI = 100 − 100 × [(PA2 − PPP)/PA2].

Statistics. For each stimulus type, results were statistically evaluated using ANOVA with one factor dose, e.g., 0, 1, 3, and 10 mg/kg for MPEP or 0 and 0.1 mg/kg for (+)-MK801 (SYSTAT 8.0). One animal (treated with vehicle) was detected as an outlier, independent of the stimulus type used. The data for this animal were excluded from the final analysis.

Results

Unconditioned Response Tests

Social Exploration. Chlordiazepoxide (5 mg/kg, p.o.), used here as a positive standard, significantly increased the time the "intruder" rat spent in active social contact when confronted with a "resident" rat (Fig. 1). Similarly, after an oral administration, MPEP in doses of 0.3 and 1 mg/kg significantly increased the duration of active social contact (Fig. 1). After 10 mg/kg MPEP, the effect was less pronounced and did not reach the level of statistical significance, potentially indicative of a bell-shaped dose-response relation. MPEP was ineffective at the very low dose of 0.003 mg/kg (p.o.).

Elevated Plus Maze. Chlordiazepoxide (10 mg/kg, p.o.), used here as a positive standard, exhibited the well known anxiolytic pattern: the time spent on open arms and the number of open arm entries was significantly increased as compared with vehicle-treated controls and this led to a significantly elevated ratio (Fig. 2, a–c). MPEP also exhibited this typical anxiolytic pattern and increased the ratio (0.1, 1, and 10 mg/kg), the number of open arm entries (0.1, 1, and 10 mg/kg), and the time spent on open arms (0.1 and 1 mg/kg, Fig. 2, a–c). However, chlordiazepoxide as well as MPEP (0.1, 1, and 10 mg/kg) increased the total number of arm entries (Fig. 2d).

Stress-Induced Hyperthermia. In the vehicle-treated cages, stress-induced hyperthermia was quantitatively comparable to the values reported in literature (+1.0°C; Fig. 3a). Chlordiazepoxide (10 mg/kg, p.o.), used here as a positive standard, significantly attenuated stress-induced hyperthermia (SIH; Fig. 3a). MPEP also induced a clear reduction in the stress-induced hyperthermia: already a dose of 7.5 mg/kg,

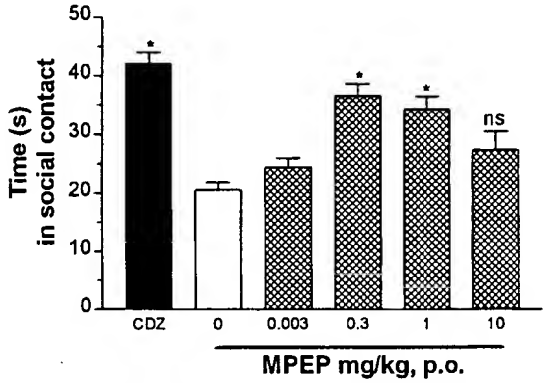

Fig. 1. Social exploration test: bars represent the mean time (seconds per 5-min trial; ± S.E.M.) during which the intruder rat actively explored the resident rat. Only the intruder rats were treated, receiving injections of MPEP [doses: 0.003 (n = 11), 0.3 (n = 11), 1 (n = 22), or 10 mg/kg, p.o. (n = 11)], chlordiazepoxide (CDZ; 5 mg/kg, p.o., n = 21) or vehicle (0 mg/kg; 0.5% methylcellulose, n = 23). Pretreatment time was 60 min. *P < .05 versus the vehicle-treated group (Dunnett's test).

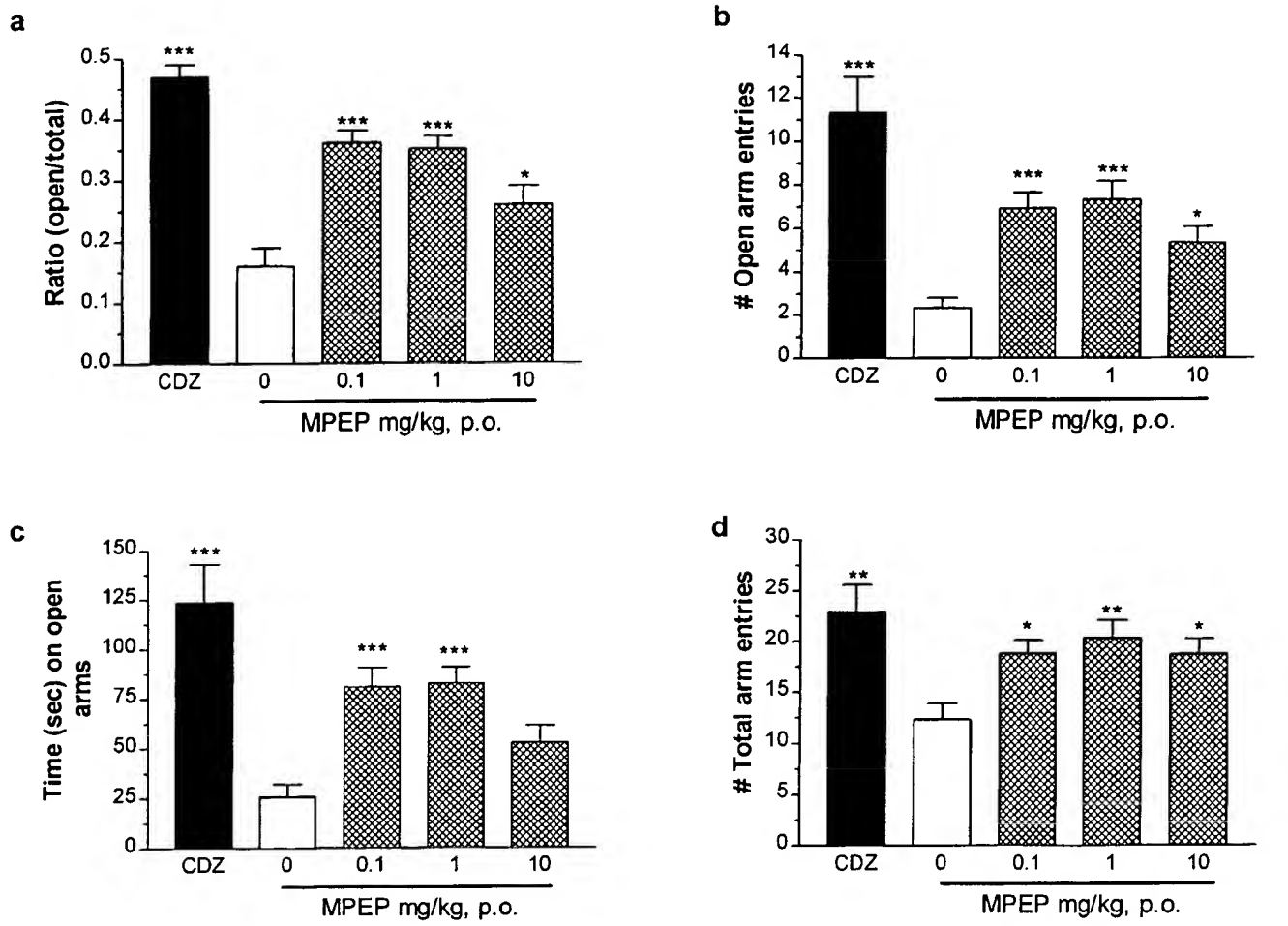

Fig. 2. Elevated plus maze: bars represent means (±S.E.M.) for the ratio (open/total arm entries) (a), the number of open arm entries (b), time (s) on open arm (c), and the total number of arm entries following treatment with MPEP (d) (doses: 0.1, 1, or 10 mg/kg, p.o.), chlordiazepoxide (CDZ; 10 mg/kg, p.o.) or vehicle (0 mg/kg; 0.5% methylcellulose). $n = 15$ per treatment group. $^{*}P < .05$, $^{**}P < .01$, or $^{***}P < .001$ versus the control-group (Dunnett's test).

p.o. tended to attenuate SIH ($P = .051$), but after a treatment with 15 and 30 mg/kg, p.o., MPEP attenuated SIH significantly (Fig. 3a). When tested at a dose of 1.5 mg/kg, p.o. (separate experiment, data not shown) MPEP was found to be ineffective. Note that none of the treatments significantly affected basal core body temperature (Fig. 3b).

Marble Burying. Mice treated with chlordiazepoxide (10 mg/kg, p.o.) buried significantly less marbles than those treated with vehicle (Fig. 4). Mice treated with MPEP also buried significantly less marbles (Fig. 4). Although the effects after treatment with 7.5 or 30 mg/kg MPEP reached the level of significance, the effect of 15 mg/kg failed to reach the level of significance (Fig. 4). Note that a low dose of 1.5 mg/kg, p.o. MPEP, which was tested in a separate experiment (data not shown), was found to be ineffective.

Conditioned Response Test

Geller-Seifter Test. Chlordiazepoxide (16 mg/kg, p.o.), i.e., the positive standard, significantly increased both the number of punished responses and the number of shocks (Fig. 5, a and b, respectively). MPEP (doses: 10, 30, or 100 mg/kg, p.o.) induced an increase in both the number of punished responses (Fig. 5a) and the number of shocks (Fig. 5b). Although the effects approached those seen with chlordiazepoxide, the response rate within the MPEP groups was too variable and, therefore, the level of statistical significance was not reached. Note that neither MPEP (10, 30, or 100 mg/kg, p.o.) nor chlordiazepoxide (16 mg/kg, p.o.) affected the number of nonpunished responses as compared with vehicle (Fig. 5c).

Locomotor Activity

Spontaneous Locomotor Activity. MPEP (doses: 0.01, 0.1, 1, 10, or 100 mg/kg, p.o.) had no effect on horizontal locomotor activity (Fig. 6a) and significantly reduced vertical activity at a dose of 100 mg/kg, p.o. only (Fig. 6b). In a separate experiment, when tested at those doses used in the SIH and marble burying study, i.e., 7.5, 15, or 30 mg/kg, p.o., MPEP was devoid of any significant effect on horizontal or vertical locomotor activity (data not shown; see also below).

***d*-Amphetamine-Induced Locomotor Activity.** Statistical significance was only found for horizontal *d*-amphetamine-induced locomotor activity (2.5 mg/kg, i.p.): all groups treated with *d*-amphetamine exhibited a significantly increased horizontal locomotor activity as compared with vehicle only (Table 1). However, no statistical significance was

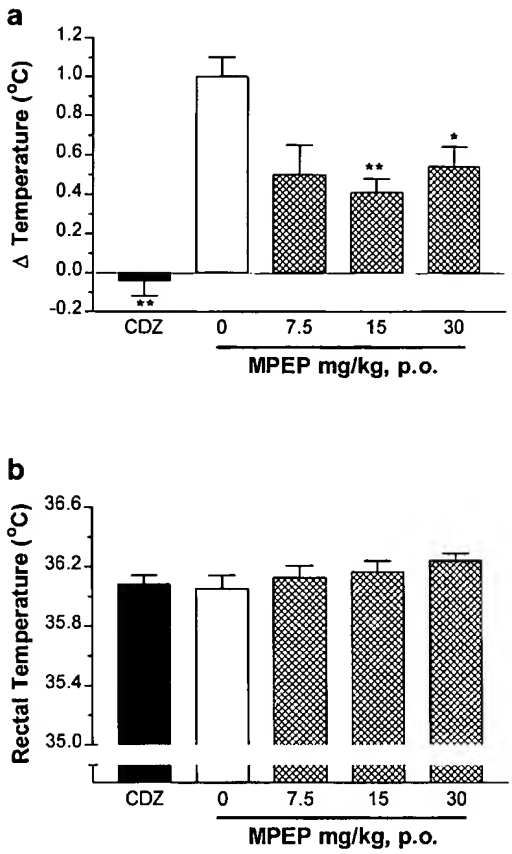

Fig. 3. Stress-induced hyperthermia: a, bars represent the mean of the Δ of the rectal temperature (±S.E.M.) per cage of 15 mice 60 min after treatment with MPEP (doses: 7.5, 15, or 30 mg/kg, p.o.; $n = 8$ per group), vehicle (0 mg/kg; 0.5% methylcellulose; $n = 6$), or chlordiazepoxide (CDZ; 10 mg/kg, p.o., $n = 6$). $^*P < .05$, $^{**}P < .01$ versus vehicle (Mann-Whitney U test). b, bars represent the mean rectal temperature (±S.E.M.) of the first mouse within the cage 60 min after treatment with MPEP (doses: 7.5, 15 or 30 mg/kg, p.o.; $n = 8$ per treatment group), vehicle (0.5% methylcellulose; $n = 6$) or chlordiazepoxide (CDZ; 10 mg/kg, p.o.; $n = 6$).

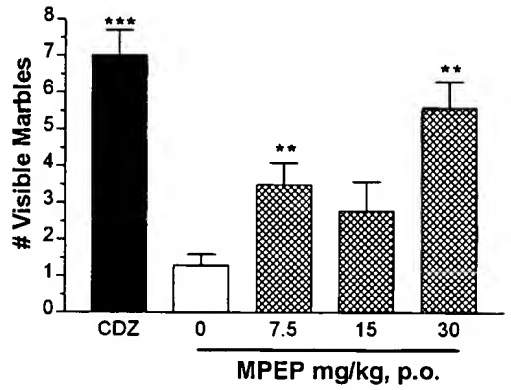

Fig. 4. Marble burying: bars represent the mean (±S.E.M.) number of marbles that were visible at the end of the 60-min trial. Mice were treated with MPEP (doses: 7.5, 15, or 30 mg/kg, p.o.; $n = 16$ per group), chlordiazepoxide (CDZ; 10 mg/kg, p.o.; $n = 12$ mice), or vehicle (0 mg/kg; 0.5% methylcellulose; $n = 12$ mice). $^{**}P < .01$, $^{***}P < .001$ versus vehicle (0 mg/kg; Mann-Whitney U test).

found for MPEP (7.5, 15, or 30 mg/kg, p.o.) or the interaction between d-amphetamine and MPEP on horizontal or vertical locomotor activity (Table 1).

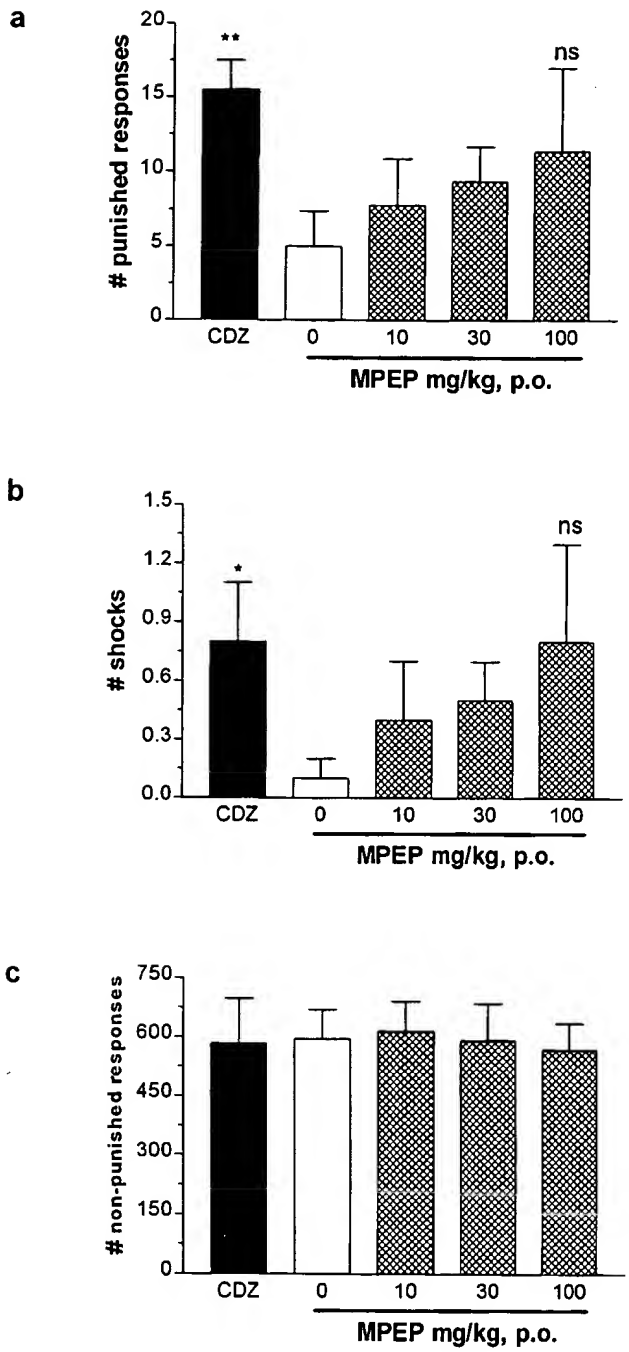

Fig. 5. Geller-Seifter test in rats: bars represent the mean number of punished responses (±S.E.M.; Fig. 1a), received shocks (Fig. 1b) and nonpunished-responses (Fig. 1c). Rats were treated with MPEP (doses: 10, 30, or 100 mg/kg, p.o.), chlordiazepoxide (CDZ; 10 mg/kg, p.o.) or vehicle (0 mg/kg; 0.5% methylcellulose). Each of the eight rats received each of the five different treatments (pretreatment time, 60 min) in a randomized order. $^*P < .05$, $^{**}P < .01$ (paired t test versus control values). ns, not significant.

Prepulse Inhibition. As expected, the ANOVA indicated a highly significant effect for (+)-MK801 (0.1 mg/kg, s.c.) on startle amplitude ($P < .001$) and on PPI ($P < .01$, $P < .001$, and $P < .001$ for prepulses of 8, 12, and 16 db above background noise, respectively). In contrast, statistical signifi-

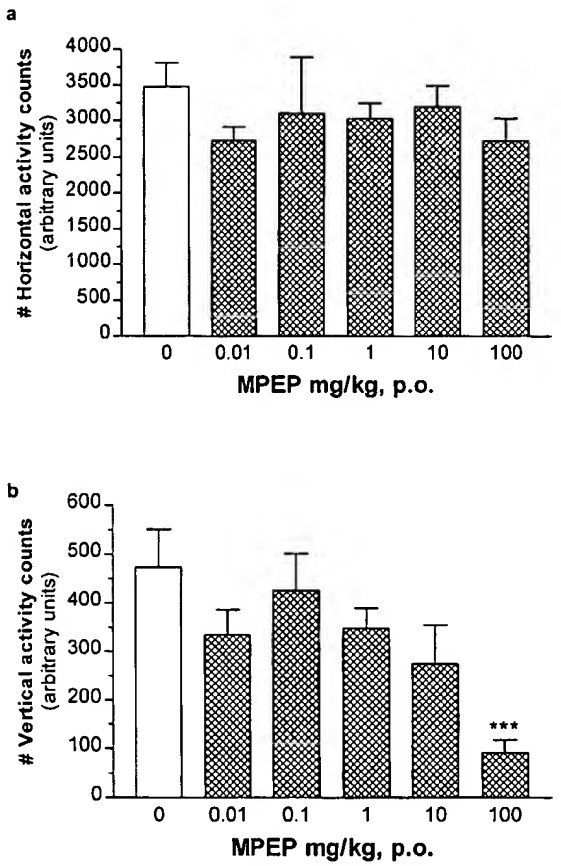

Fig. 6. Locomotor activity: bars represent the mean (±S.E.M.) number of horizontal (a) and vertical (b) activity counts in 120 min of registration after treatment with MPEP (doses: 0.01, 0.1, 1, 10, or 100 mg/kg, p.o.) or vehicle (0 mg/kg; 0.5% methylcellulose). n = 15 per treatment group.

TABLE 1

d-Amphetamine-induced locomotor activity

Values represent the mean (±S.E.M.) number of total horizontal and vertical activity counts (arbitrary units) in 120 min of registration after treatment with MPEP (doses: 7.5, 15, and 30 mg/kg, p.o.), vehicle only (methylcellulose, 0.5%), or in their respective combination with *d*-amphetamine (2.5 mg/kg, i.p.).

Treatment		n	Activity Counts ± S.E.	
MPEP (p.o.)	*d*-Amphetamine (i.p.)		Horizontal	Vertical
mg/kg			*arbitrary units*	
0	0	15	14.7 ± 3.2	95.5 ± 29.7
7.5	0	15	20.0 ± 4.5	123.5 ± 33.5
15	0	15	15.5 ± 3.1	59.4 ± 20.8
30	0	15	32.8 ± 10.6	220.7 ± 90.5
0	2.5	15	83.1 ± 15.1***	123.1 ± 27.0
7.5	2.5	15	69.1 ± 8.5***	77.3 ± 20.9
15	2.5	15	90.5 ± 14.9***	71.5 ± 24.7
30	2.5	15	80.6 ± 14***	103.2 ± 45.6

*** $P < .001$ versus vehicle only.

cance was found for MPEP (1, 3, or 10 mg/kg, p.o.) neither on startle amplitude ($P > .05$) nor the PPI ($P > .05$; Table 2).

Discussion

The recently identified selective and systemically active antagonists for the $mGlu_5$ receptor have made possible the experimental study of the consequences of a blockade of this receptor subtype in behavior as well as the effect of high affinity ligands as a potential treatment of disease states (Gasparini et al., 1999; Varney et al., 1999). Because MPEP is one of the most potent derivatives within this series of drugs, this compound was tested in various rodent models of anxiety. These animal models of anxiety can be differentiated into two main categories, the so-called conditioned response and the so-called unconditioned response paradigms (Rodgers, 1997; Rodgers and Dalvi, 1997; Olivier et al., 2000). MPEP was tested in several unconditioned response tests (social exploration test, elevated plus maze, stress-induced hyperthermia, and marble burying) and in one conditioned response test (Geller-Seifter test). The present data indicate that MPEP can exhibit anxiolytic-like activity in several rodent models of anxiety.

To test the prototypical representative of this new class of compounds as thoroughly as possible, MPEP was tested in a variety of standard, unconditioned test paradigms (Olivier et al., 2000). The tests used here can be differentiated and described as a model of "social anxiety" (assessed in the social exploration test in rats), a model of "novelty-induced" anxiety (assessed in the marble burying test), a model of anxiety in an "approach-avoidance conflict" (assessed in the elevated plus maze), and finally a model of "anticipatory anxiety" (assessed in the stress-induced hyperthermia paradigm in mice). In all these unconditioned paradigms, MPEP significantly and positively modulated the "anxiety"-dependent variable: anxiolytic-like effects were seen in the social exploration test and in the elevated plus maze in rats. The latter findings could also be confirmed in mice (C. Gentsch, unpublished observation). Given that MPEP, at doses between 0.01 and 30 mg/kg, p.o., did not alter horizontal or vertical activity in mice when exposed to a novel environment, it is unlikely that an effect on activity induced by MPEP has biased these findings. However, it is important to note that MPEP significantly increased the total number of arm entries, i.e., an indication of increased activity, although the effect was less pronounced as that seen for chlordiazepoxide. Accordingly, the effect of MPEP, as seen in these animal models, is indeed most likely to reflect anxiolysis. The same line of argumentation can be used in the SIH paradigm in mice. The principle, i.e., hyperthermia induced by anticipatory anxiety, is also a recognized and well described phenomenon in humans (Reeves et al., 1985), and autonomic (dys)function is one of the items in the diagnosis of generalized anxiety disorders (*Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition*). The effect of MPEP on stress-induced hyperthermia can be considered as specific, because the compound did not affect the core temperature per se: obviously, MPEP selectively counteracted the anxiety-dependent variable. It is worthwhile to note that this particular test differs from the other "behavioral" unconditioned response tests in that SIH is hypothesized to model autonomic reflexes, which are triggered by emotional activation. It is suggested that such reflexes exist in various forms of anxiety and potentially represent a relatively common expression of anxiety (Lecci et al., 1990).

In the conditioned response paradigm, i.e., the Geller-Seifter test, an increase was found for the number of punished responses and shocks but, in contrast to the effect found after treatment with chlordiazepoxide, the effect of MPEP failed to reach significance in both variables; obviously, the higher variability in those groups treated with

TABLE 2

Prepulse inhibition

Values represent the mean (±S.E.M.) effect of a treatment with MPEP (1, 3, or 10 mg/kg; pretreatment time, 60 min), (+)-MK801 (0.1 mg/kg; pretreatment time, 30 min), or their respective vehicles, i.e. 0.5% methylcellulose or saline, on startle amplitude (g) and prepulse inhibition (%).

Compound	Dose	Startle Amplitude			Prepulse Inhibition			
		PA1	PA2	PA3	PPP, 8 db	PPP, 12 db	PPP, 16 db	N
	mg/kg		g			%		
MPEP (p.o., 60 min)	0	196 ± 19	154 ± 25	147 ± 32	38 ± 6	60 ± 2	83 ± 4	7
	1	180 ± 22	172 ± 20	141 ± 28	47 ± 2	61 ± 5	90 ± 2	8
	3	199 ± 22	200 ± 15	168 ± 20	39 ± 7	57 ± 4	88 ± 2	8
	10	197 ± 23	196 ± 26	166 ± 12	43 ± 4	57 ± 6	90 ± 3	8
P		>.05	>.05	>.05	>.05	>.05	>.05	
(+)-MK801 (s.c., 30 min)	0	202 ± 28	186 ± 26	140 ± 35	31 ± 5	53 ± 4	86 ± 3	8
	0.1	579 ± 29	526 ± 20	497 ± 22	7 ± 5	18 ± 5	33 ± 5	8
P		<.001	<.001	<.001	<.01	<.001	<.001	

MPEP (particularly at the 100 mg/kg dose) was fundamental to these statistical findings. The reasons for the higher variability as compared with their reaction to chlordiazepoxide are at present unclear but might be explained by the fact that the animals were preselected per se on their positive response to chlordiazepoxide (see *Materials and Methods*) in combination with the relatively low number of rats per group. Preliminary findings in two other conditioned response tests, i.e., fear potentiated startle (M. Koch et al., oral communication) and the Vogel test (A. Pilc et al., oral communication), suggest that MPEP exhibits anxiolytic effects in this type of tests. It should be mentioned, however, that MPEP has analgesic effects in inflammatory pain models in rats (Walker et al., 2000a,b), and, accordingly, differences in shock perception may (partially) influence the behavioral response in conditioned test paradigms.

Given the high affinity and selectivity of MPEP for $mGlu_5$ receptors as outlined in the introduction, it is safe to assume that the effects are indeed mediated by inhibition at this glutamate receptor. The mechanism of action of MPEP in relation to its anxiolytic effect is at present unclear. The fact that MPEP neither potentiated nor inhibited *d*-amphetamine-induced locomotor activity (this study) or apomorphine-induced climbing (W. P. J. M. Spooren, unpublished observation) may indicate that the effect does not involve directly or indirectly dopamine or one of its receptors, at least in the nonlesioned brain (however, see also Spooren et al., 2000). This latter observation is, for example, in contrast to buspirone, which has been shown to have a dopaminergic (antagonistic) component (Koek et al., 1998). Obviously, the exact function of $mGlu_5$ receptors in behavior and anxiety remains to be further elucidated in future, additional studies.

The present study used a variety of different paradigms, each of which is known to model or reflect different forms/aspects of anxiety. These different behavioral components are known to be modulated by anxiolytic drugs, as reported in their respective pharmacological validation. With regard to active doses, it is a well known fact that the same compound may act at different dose ranges in distinct models (Olivier et al., 2000). The effect of MPEP is in this respect no exception: anxiolytic doses of MPEP were variable in distinct models. However, given the variety of tests and dose ranges used here, a relatively good estimation on the optimal anxiolytic dose range has been obtained and can be proposed for future experimental as well as for clinical studies.

Two final points: 1) All tests described here were performed after a single administration. Given the fact that in humans anxiolytic drugs are administered repeatedly it remains to be determined as to whether the MPEP-induced effects will be retained after subchronic administration. 2) From clinical experience it is well known that some of the widely used anxiolytics induce unwanted side effects such as amnesia, interaction with alcohol, or unfavorable withdrawal symptoms after an abrupt cessation of a long-term treatment. At present it is unknown whether MPEP can be favorably distinguished with regard to its efficacy and/or its side effect profile from the most frequently used anxiolytics. However, the present study indicated that MPEP up to a dose of 100 mg/kg induced no marked sedation in mice. In addition, MPEP had no effect on PPI, which is indicative for the absence of psychotomimetic side effects, i.e., one of the major drawbacks that plagued the ionotropic *N*-methyl-D-aspartate receptor antagonists (Danysz et al., 1996).

In summary, this present set of data is clearly indicative of a potential anxiolytic activity of $mGlu_5$ receptor antagonists. The novel mechanism and the potential absence of sedation and psychotomimetic effects as assessed in the spontaneous locomotor activity and PPI paradigm, suggest that $mGlu_5$ receptor antagonists may indeed represent a new and safe approach for the treatment of anxiety.

Acknowledgments

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Multiple MPEP administrations evoke anxiolytic- and antidepressant-like effects in rats

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Abstract

Several lines of evidence suggest a crucial involvement of glutamate in the mechanism of action of anxiolytic and antidepressant drugs. The involvement of group I mGlu receptors in anxiety and depression has also been proposed. In view of the recent discovery of anxiolytic- or antidepressant-like effects of acute injections of 2-methyl-6-(phenylethynyl)-pyridine (MPEP), a selective and brain penetrable mGlu5 receptor antagonist, we designed the present study to examine anxiolytic- and/or antidepressant-like effects of multiple administrations of this drug. The anxiolytic-like effects of MPEP were evaluated in rats using the conflict drinking test. The antidepressant-like effect was estimated using the rat olfactory bulbectomy model of depression. Seven subsequent injections of MPEP (1 mg/kg) significantly (by 320%) increased the number of shocks accepted during the experimental session in the Vogel test. MPEP given once daily at a dose of 10 mg/kg, restored the learning deficit of bulbectomized rats after 14 days of treatment, remaining without any effect in the sham-operated animals. *N*-methyl-D-aspartic acid (NMDA)-induced convulsions in mice were not affected by a single injection of MPEP (30 mg/kg) indicating that at this dose MPEP did not block NMDA receptors. The results indicate that the prolonged blockade of mGlu5 receptors exerts anxiolytic- and antidepressant-like effects in rats. No tolerance to anxiolytic-like action occurs. The previously mentioned results further indicate that antagonists of group I mGlu receptors may play a role in the therapy of both anxiety and depression.

Keywords: Animal models of depression; Anxiety; Conflict drinking test; Convulsions; Depression; mGlu5 receptors; 2-Methyl-6-(phenylethynyl)-pyridine; Olfactory bulbectomy; *N*-methyl-D-aspartic acid

1. Introduction

Excitatory amino acids (EAA) are abundant in the brain. It has been estimated that ca. 50% of neurons in the mammalian brain may utilize glutamate as a neurotransmitter (Mc Geer et al., 1987). Glutamate, the most abundant EAA in the brain, acts by stimulation of ionotropic and metabotropic glutamate receptors (Monaghan et al., 1989; Conn and Pin, 1997). Ionotropic glutamate receptors are coupled ion channels and are classified as *N*-methyl-D-aspartic acid (NMDA), AMPA and kainate receptors (Monaghan et al., 1989). Metabotropic glutamate receptors (mGluR) are the members of a relatively new class of glutamate receptors linked to G-proteins. Eight different subtypes of mGlu receptors have been identified so far (mGlu1–8). On the basis of their sequence homology, effector coupling and pharmacology, mGlu receptors have been subdivided into three groups: group I mGlu receptors (mGlu1 and mGlu5), positively coupled to phospholipase C; group II mGlu receptors (mGlu2 and mGlu3) and group III mGlu receptors (mGlu4, mGlu6, mGlu7 and mGlu8), negatively coupled to adenylate cyclase (Pin and Duvoisin, 1995).

Glutamate seems to play a major role in both physiology and pathophysiology of the central nervous system. Some data show that changes in the ionotropic glutamate neurotransmission may be involved in a variety of neuropsychiatric disorders (Wróblewski and Danysz, 1989; Danysz et al., 1996). Converging lines of evidence

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indicate crucial involvement of glutamate receptors in the phenomena related to the mechanism of action of anxiolytic drugs (for review see: Wiley et al., 1995; Chojnacka-Wójcik et al., 2001) or antidepressant drugs (Skolnick et al., 1996, 2001; Skolnick, 1999). The recent clinical study demonstrates an antidepressant effect of ketamine, an uncompetitive NMDA antagonist (Berman et al., 2000). The hope that ionotropic glutamate receptor antagonists, mainly NMDA receptor blockers, could be applied in the therapy of CNS disorders, was hampered by the fact that these drugs produce pronounced, undesired side-effects such as psychotomimetic effects, memory impairment and ataxia, which were demonstrated in preclinical studies (see Danysz et al., 1996). The use of substances that modulate nervous system function, such as the metabotropic glutamate receptor ligands may be one of the possible solutions to this problem.

An involvement of group I mGlu receptors in psychiatric disorders such as depression and anxiety has been suggested (for review see Chojnacka-Wójcik et al., 2001; Pilc et al., 2002). Chronic antidepressant treatment influences expression and function of group I mGlu receptors in the hippocampus (Bajkowska et al., 1999; Pilc et al., 1998). It has been shown that antagonists of group I mGlu receptors evoke anxiolytic-like effects after intrahippocampal injection in rats (Chojnacka-Wójcik et al., 1997; Zahorodna and Bijak, 1999). The 2-methyl-6-(phenylethynyl)-pyridine (MPEP), is a potent, noncompetitive antagonist of mGlu5 receptors with an IC_{50} of 36 nM at the human mGlu5a receptor in the PI hydrolysis assay (Gasparini et al., 1999). A single dose of MPEP exhibited anxiolytic-like effects in several animal tests (Kłodzińska et al., 2000; Spooren et al., 2000; Tatarczyńska et al., 2001). Antidepressant-like effects of that compound were also described (Tatarczyńska et al., 2001). Since the treatment of anxiety and depression requires long-term drug administration, we decided to investigate whether tolerance develops to anxiolytic and antidepressant effects of MPEP after prolonged treatment. The present results indicate that MPEP shows anxiolytic- and antidepressant-like effects after multiple drug administrations. Acute experiments performed on mice, demonstrating that MPEP at 30 mg/kg has no influence on NMDA-induced convulsions, exclude the possibility of the blockade of NMDA receptors by MPEP.

2. Materials and methods

2.1. Animals

The experiments with multiple MPEP administration concerning its anxiolytic- and antidepressant-like effects were performed on male Wistar rats (200–250 g), while the acute studies testing the influence of MPEP on NMDA-induced convulsions, were performed on male C57BL/6J mice (23–26g). The animals were kept on a natural day–night cycle at room temperature between 19–21 °C, with free access to food and water. Each experimental group consisted of 6–10 naive animals per drug dose. Injection volume was 2 ml/kg in rats and 10 ml/kg in mice. Experiments were carried out between 9:00 a.m. and 2:00 p.m. by an observer unaware of the treatment. All experimental procedures were approved by Animal Care and Use Committee at the Institute of Pharmacology, Polish Academy of Sciences in Krakow.

2.2. Conflict drinking test (Vogel test)

The test was performed according to the modified method of Vogel et al. (1971) described subsequently. On the first day of the experiment, the rats were adapted to the test chamber for 10 min. It was a Plexiglas box ($27 \times 27 \times 50 cm^3$), equipped with a grid floor made of stainless steel bars and a drinking bottle with tap water. After the adaptation period, the animals were deprived of water for 24 h, and were then placed in the test chamber for another 10-min adaptation period during which they had a free access to the drinking bottle. Afterwards, they were allowed a 30-min free-drinking session in their home cage. After another 24 h water deprivation period, the rats were again placed in the test chamber and were allowed to drink for 30 s. Immediately afterwards, drinking was punished with electric shock (0.5 mA). The electric impulses between the grid floor and the spout of the drinking bottle were released every 2 s (timed from the moment when a preceding shock was delivered). Each shock lasted for 1 s and if the rat was drinking when an impulse was released, it received a shock. The number of shocks obtained during a 5-min experimental session was recorded. MPEP (1 mg/kg) and diazepam (10 mg/kg) or saline were administered once daily for 6 days. Twenty-four hours after the last injection, a challenge dose of these drugs or saline was administered.

2.3. Olfactory bulbectomy: surgical procedure

Two weeks after arrival at the laboratory, bilateral olfactory bulbectomy (OB) was performed under equithesin anesthesia in rats. Following exposure of the skull, burr holes were drilled 7 mm anterior to the bregma and 2 mm on either side of the middle line at a point corresponding to the posterior margin of the orbit of the eye. The olfactory bulbs were removed by suction and the burr holes were filled with hemostatic sponge. The skin was closed. Sham-operated animals were treated in the same way, but the olfactory bulbs were left intact. The animals were allowed to recover for 14 days following surgery, and they were handled daily by the exper-

imenter throughout the recovery period to eliminate any aggressiveness that would otherwise arise.

2.4. Passive avoidance test

The apparatus consisted of an open box ($55 \times 55 \times 55 cm^3$) with black walls and stainless steel grid floor. The rods were 1.2 cm apart and were connected to the terminals of a stimulator delivering square wave pulse with a constant voltage. The delivered shock had constant intensity (0.75 mA) and lasted 1s. A wooden platform measuring $12 \times 12 \times 4 cm^3$ was in the center of the box. Each rat was placed on this platform and when it left the platform with all four paws it received an electric shock. The animal was immediately removed from the experimental cage and transferred to its home cage. The next trial was initiated 30 s thereafter. The training of the rat was stopped if the rat learned to stay still on the platform for 1 min or if 15 trials were carried out, as some of the rats never learned the contingency. Two weeks following surgery repeated drug treatment began. MPEP or desipramine (DMI, 10 mg/kg each) was administered once daily for 14 days. The last dose was given 45 min before the passive avoidance test. Control animals received injections of sterile saline.

2.5. NMDA-induced convulsions

NMDA (125 mg/kg)-induced convulsions were evoked 30 min after MPEP administration. Each animal was observed for 60 min, starting immediately after convulsant drug injection. The number of animals with clonic convulsions and mortality was recorded.

2.6. Drugs

Diazepam (Polfa-Poznań, Poland) was suspended in a 1% aqueous solution of Tween 80. MPEP (Novartis, Basle), DMI (Sigma), and NMDA (Sigma) were dissolved in sterile saline. All substances were administered intraperitoneally (i.p.). The correct vehicle was used as a control for each treatment.

2.7. Analysis of the data

The data are presented as means ± SEM. Statistical significance of the results was evaluated by one-way analysis of variance followed by Dunnett's Multiple Comparison Test (Table 1) or Newman–Keuls test (Fig. 1), using GRAPHPAD PRISM software, with $P < 0.05$ considered significant. The seizure data were analyzed with the help of Fisher's two tailed, exact probability test.

Table 1
Effect of single or multiple treatment with MPEP or diazepam (Diaz) in the conflict drinking test in rats

Multiple dose (6 days)	Challenge dose	n	Number of shocks obtained/5 min
Vehicle	Vehicle	8	9.5 ± 1.28
Vehicle	MPEP	8	47.3 ± 6.60*
MPEP	MPEP	8	39.0 ± 7.40*
Vehicle	Diazepam	8	52.8 ± 5.70*
Diazepam	Diazepam	8	60.5 ± 7.2*
			$F(4,33) = 83.1, P < 0.0001$

Rats were injected (i.p.) with vehicle, MPEP or diazepam for 6 days and challenged with vehicle, MPEP or diazepam 24 h later. The test was performed at the end of the experiment, 45 min after the last dose of drugs. Values are expressed as means ± SEM, n is the number of rats per group. Symbols indicate significance of differences in Dunnett's Multiple Comparison Test $*P < 0.001$ vs vehicle treated rats.

3. Results

3.1. Conflict drinking test in rats

MPEP at a single dose of 1 mg/kg i.p. (which in our earlier studies induced a clear anxiolytic-like effect (Tatarczynska et al., 2001) significantly ($F(4,33) = 83.1, P < 0.0001$) increased the number of shocks (by 497%) accepted during the experimental session in the Vogel test (Fig. 1). This effect was comparable to that seen after single dose of diazepam (10 mg/kg), which was used as a reference drug. Seven injections of MPEP (once daily for 7 days) significantly (by 410%) increased the number of shocks accepted in this test (Table 1). This effect was again comparable to that seen after multiple doses of diazepam (10 mg/kg).

3.2. Antidepressant-like effect of MPEP

As shown in Fig. 1, sham-operated animals learned the passive avoidance procedure in approximately four trials when tested 4 weeks after the surgery (2 weeks after chronic saline treatment). Rats with bilateral olfactory bulb ablation needed an average of nine trials to fulfill this criterion. MPEP, an antagonist of mGluR5 receptors, given once daily at a dosage of 10 mg/kg, restored the learning capacity in bulbectomized rats after 14 days of treatment (Fig. 1A), remaining without any effect in the sham-operated animals ($F(3,28) = 3.975, P < 0.05$). The dose of MPEP was chosen on the basis of our earlier experiments (Tatarczynska et al., 2001). DMI, an antidepressant drug used as a reference substance reduced the effect of bulbectomy and had no influence on behavior of sham-operated animals (Fig. 1B) ($F(3,31) = 5.447, P < 0.01$). In the case of single

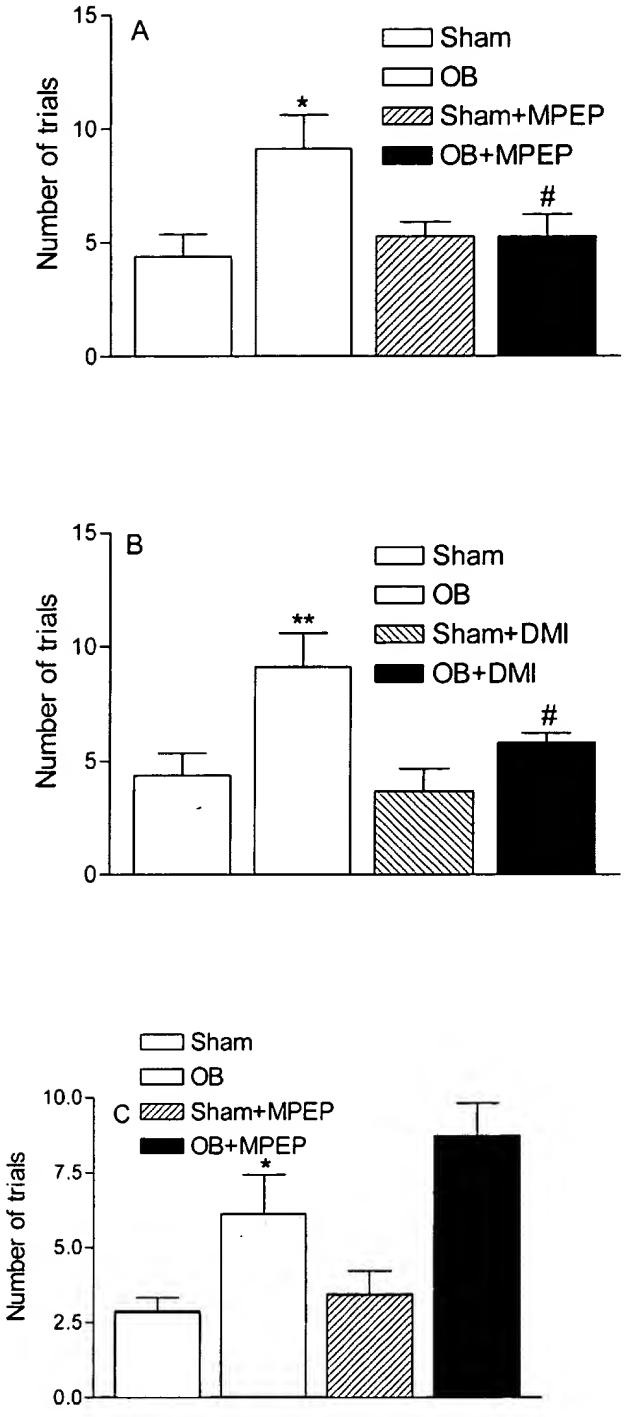

Fig. 1. Effects of OB, MPEP and DMI on the passive avoidance procedure. A. Effect of multiple MPEP administration. B. Effect of multiple DMI administration. C. Effect of single MPEP administration. MPEP or DMI was administered 2 weeks after OB for 14 days, i.p. The test was performed 45 min after the last dose of drugs. Values are expressed as means ± SEM, n = 7–8. ANOVA as follows: Upper panel—($F(3,28) = 3.975, P < 0.05$). Middle panel—($F(3,31) = 5.447, P < 0.01$). Lower panel—($F(3,28) = 7.143, P < 0.05$). Symbols indicate significance of differences in Newman–Keuls test. $*P < 0.05$ vs sham-operated animals, $**P < 0.01$ vs sham-operated animals, # $P < 0.05$ vs OB animals.

drug administration MPEP was ineffective in restoring learning capacity (Fig. 1C) ($F(3,28) = 7.143, P < 0.05$).

3.3. NMDA-induced convulsions

MPEP at a dose of 30 mg/kg did not prevent NMDA (125 mg/kg)-induced convulsions in mice. All the 10 NMDA treated mice exhibited convulsions while in the MPEP pretreated animals the number was nine out of 10 (the seizure data were analyzed with the help of Fishers two tailed, exact probability test using GraphPad Prism software). MPEP (30 mg/kg) did not also affect mortality induced by NMDA in mice. In NMDA-administered group, all 10 tested mice died, while in MPEP pretreated group nine out of 10 died. The inability of MPEP to inhibit NMDA-induced convulsions, indicates that this compound at a dose of 30 mg/kg has no antagonistic action on population of NMDA receptors.

4. Discussion

4.1. Anxiety

Depression and anxiety are recurrent, very chronic and disabling mental disorders, which account for half of the burden of mental disorders (Vos and Mathers, 2000). The prevalence of depression has increased significantly during the last 50 years, from less than 1% in the 1950s to 10–15% of the population in the 1990s (Healy, 1999). The prevalence of anxiety is also rising and may reach half of the value for depression (Andrews et al., 2000; Barrett et al., 1988). Majority of the patients with a principal diagnosis of unipolar major depressive disorder have a comorbid anxiety disorder (Zimmerman et al., 2000). Hence, it is important to develop compounds with both antidepressant and anxiolytic actions. Our earlier data have shown that (*S*)-4-carboxy-3-hydroxyphenylglycine (*S*-4C3H-PG), an antagonist of group I mGluR, exhibits anxiolytic-like activity in animals after intrahippocampal administration (Chojnacka-Wójcik et al., 1997). MPEP, the selective antagonist of the mGlu5 receptor produced a prominent anxiolytic-like activity in so-called conditioned response tests such as Vogel test and the four plate test (Kłodzińska et al., 2000; Tatarczyńska et al., 2001) and in the unconditioned response test such as elevated plus maze test, novelty-induced anxiety and social anxiety (Spooren et al., 2000; Tatarczyńska et al., 2001). However, all the effects were described after single drug dose. Therefore, we decided to investigate whether multiple MPEP administrations can also produce anxiolytic-like effects. The present results show that 1 week of MPEP or diazepam treatment is without any influence on their ability to induce anxi-

olytic-like effects, indicating the lack of tolerance to that effect of both drugs.

Despite the fact that MPEP binding sites are located postsynaptically, this compound has recently been reported to inhibit glutamate release (Thomas et al., 2001) via presynaptically located mGluR5 receptors (Thomas et al., 2000). This action resembles the function of group II mGluRs, which are predominantly located presynaptically and function to modulate glutamate release (Schoepp, 2001). Group II mGlu receptor agonists, such as LY354740, which modulate the release of glutamate, cause anxiolytic-like effects in animals (Helton et al., 1998; Kłodzińska et al., 1999). Whether the purported influence of MPEP on glutamate release contributes to its anxiolytic-like effects remains to be investigated. The modulation of NMDA receptor sensitivity by MPEP (see Section 4.2) has also to be taken into account as the possible mechanism of its anxiolytic-like effects. It is well known that NMDA receptor antagonists induce anxiolytic-like effects in animals (for a recent review see Chojnacka-Wójcik et al., 2001). However, tolerance develops to anxiolytic effects of some functional NMDA receptor antagonists such as ACPC (Przegaliński et al., 1999), while no tolerance is seen after multiple MPEP treatment. Irrespective of its mode of action MPEP seems to decrease the overall glutamatergic neurotransmission. The decreased glutamatergic transmission, which leads to overall inhibitory effects in the central nervous system may have consequences similar to the effects of the increased GABA-ergic transmission, which is considered to underlie anxiolytic effects of benzodiazepines (Haefely et al., 1975).

4.2. Depression

Previously published data demonstrated that a single dose of MPEP did shorten the immobility time in a tail suspension test in mice, which shows its high predictive validity for identifying potentially useful pharmacotherapies of depression. In the present study bilateral OB was chosen to evaluate the effects of prolonged MPEP administration. Bilateral OB in the rat is associated with neurochemical, physiological and behavioral changes, resembling the symptoms observed in depressed patients (Jesberger and Richardson, 1985). These symptoms are reversed by chronic, but not acute, treatment with antidepressant drugs (Cairncross et al., 1979; Jancsar and Leonard, 1984; Leonard and Tuite, 1981; Lloyd et al., 1983). Other classes of drugs used in the treatment of psychiatric disorders, such as anxiolytics and antipsychotics, do not reverse bulbectomy-induced behavioral deficits (van Riezen et al., 1977). OB model of depression can detect the antidepressant-like activity of several classes of antidepressant drugs, including substances, which do not act on the monoaminergic systems in the brain (Lloyd et al., 1983). Multiple (but not single) MPEP administrations restored normal behavioral patterns in the olfactory bulbectomized rats, in a manner similar to classical antidepressant drug—DMI, used in this study as a positive control. MPEP was reported not to change exploratory activity; therefore sedation or motor impairments are not responsible for the effect of the substance (Tatarczyńska et al., 2001).

Preclinical data indicate that compounds, which reduce transmission at NMDA receptors, display antidepressant-like activity (Skolnick, 1999). Glutamatergic transmission by the stimulation of group I mGlu receptors has been shown to potentiate the ionotropic glutamate receptor-mediated responses in various experimental models (Glaum and Miller, 1993, 1994), including potentiation of NMDA currents (Fitzjohn et al., 1996; Ugolini et al., 1999). It was reported that mGluR5 antagonists reduced NMDA receptor activity in several brain areas (Awad et al., 2000; Attucci et al., 2001; Doherty et al., 1997; Pisani et al., 2001). Therefore, reduction in glutamatergic transmission via NMDA receptors induced by MPEP may contribute to the antidepressant-like effect of MPEP. Direct blockade of NMDA receptors by MPEP can be excluded as the substance inhibited the NMDA receptor activity at a concentration higher than 10 μM (Oleary et al., 2000), which exceeds 1000 times its IC_{50} for inhibition of mGlu5 receptor activity (Gasparini et al., 1999) [for recent discussion see Spooren et al., 2001]. MPEP at a dose of 30 mg/kg, i.e. three times higher than the dose used in chronic experiments, neither inhibited NMDA-induced convulsion, nor did it prevent animal's mortality, indicating that at the dosages used in our experiments it is without any influence on NMDA receptors. Therefore, the data indicate that the antidepressant-like effect of MPEP is due to the blockade of mGlu5 receptors.

The preceding results further indicate that antagonists of group I mGlu receptors may play a role in the therapy of both anxiety and depression. Combined anxiolytic- and antidepressant-like actions of MPEP may be of potential importance for the discovery of future antidepressant and anxiolytic drugs.

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Potential anxiolytic- and antidepressant-like effects of MPEP, a potent, selective and systemically active mGlu5 receptor antagonist

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1 Several lines of evidence suggest a crucial involvement of glutamate in the mechanism of action of anxiolytic and/or antidepressant drugs. The involvement of group I mGlu receptors in anxiety and depression has also been proposed. Given the recent discovery of a selective and brain penetrable mGlu5 receptor antagonists, the effect of 2-methyl-6-(phenylethynyl)-pyridine (MPEP), i.e. the most potent compound described, was evaluated in established models of anxiety and depression.

2 Experiments were performed on male Wistar rats or male Albino Swiss or C57BL/6J mice. The anxiolytic-like effects of MPEP was tested in the conflict drinking test and the elevated plus-maze test in rats as well as in the four-plate test in mice. The antidepressant-like effect was estimated using the tail suspension test in mice and the behavioural despair test in rats.

3 MPEP (1–30 mg kg^{-1}) induced anxiolytic-like effects in the conflict drinking test and the elevated plus-maze test in rats as well as in the four-plate test in mice. MPEP had no effect on locomotor activity or motor coordination. MPEP (1–20 mg kg^{-1}) did shorten the immobility time in a tail suspension test in mice, however it was inactive in the behavioural despair test in rats.

4 These data suggest that selective mGlu5 receptor antagonists may play a role in the therapy of anxiety and/or depression, further studies are required to identify the sites and the mechanism of action of MPEP.

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Keywords: mGlu5 receptors; MPEP; conflict drinking test; four-plate test; plus-maze test; tail suspension test; anxiety; depression

Abbreviations: S-4C3H-PG, (S)-4-Carboxy-3-hydroxyphenylglycine; CNS, central nervous system; GABA, γ-aminobutyric acid; L-5-HTP, L-5-hydroxytryptophan; mGluR, metabotropic glutamate receptors; MPEP, 2-methyl-6-(phenylethynyl)-pyridine; NMDA, N-methyl-D-aspartic acid

Introduction

Glutamate is the major excitatory neurotransmitter in the brain, and as such involved in several physiological and pathological conditions (Wroblewski & Danysz, 1989; Danysz *et al.*, 1996). Glutamate acts at two classes of receptors, the ionotropic and the metabotropic glutamate receptors (mGlu receptors) (Monaghan *et al.*, 1989; Conn & Pin, 1997). Metabotropic glutamate receptors are a family of eight G-protein coupled receptors which are classified into three groups according to their sequence homology, effector coupling and pharmacology. Group I mGlu receptors (mGlu1 and mGlu5) are positively coupled to phospholipase C; group II mGlu receptors (mGlu2 and mGlu3) and group III mGlu receptors (mGlu4, mGlu6, mGlu7 and mGlu8) are negatively coupled to adenylate cyclase (Conn & Pin, 1997). Activation of group I mGlu receptors leads to a transient increase in intracellular calcium *via* the production of inositol-trisphosphates (Conn & Pin, 1997). Generally, it has been shown that activation of group I receptors enhances or facilitates the excitatory effects of glutamate by modulation of ion channel activity (Conn & Pin, 1997). Antagonists of group I mGlu receptors have been proposed to exhibit potential positive therapeutic effects (Bruno *et al.*, 1994; Conn & Pin, 1997) in CNS disorders related to excessive excitatory neurotransmission such as epilepsy, ischaemia and pain (Nicoletti *et al.*, 1996; Conn & Pin, 1997).

Several lines of evidence suggest an important role for glutamate in anxiety and depression (Wiley *et al.*, 1995; Skolnick *et al.*, 1996; Danysz & Parsons, 1998; Skolnick, 1999). Involvement of group I mGlu receptors in psychiatric conditions such as depression and anxiety has also been proposed. It has been shown that antagonists of group I mGlu receptors exert anxiolytic-like effects after intrahippocampal injection in rats (Chojnacka-Wójcik *et al.*, 1997); and that antidepressant treatment influences group I mGlu receptors in the hippocampus (Bajkowska *et al.*, 1999; Pilc *et al.*, 1998).

Up to now studies concerning involvement of mGlu5 receptors in CNS functions were largely based on compounds which have only limited selectivity between mGlu1 and mGlu5 receptor subtypes (Nicoletti *et al.*, 1996; Conn & Pin,

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1997) and which do not penetrate into the brain. Only recently, novel, selective and systemically active compounds have been described (Varney *et al.*, 1999; Gasparini *et al.*, 1999). The most potent of this series is 2-methyl-6-(phenylethynyl)-pyridine (MPEP), a noncompetitive antagonist with an IC_{50} of 36 nM at the human mGlu5a receptor in the PI hydrolysis assay but no significant effect at other metabotropic or ionotropic glutamate receptors (Gasparini *et al.*, 1999). To evaluate whether MPEP has anxiolytic-or antidepressant-like effects, we studied its effects in several models of anxiety or depression in rats and mice.

Methods

Animals and housing

The experiments were performed on male Wistar rats (200–250 g) and male Albino Swiss or male C57BL/6J mice (22–26 g). The animals were kept on a natural day–night cycle at a room temperature of 19–21°C, with free access to food and water. Each experimental group consisted of 6–10 naïve animals/dose. In rats, all injections were given in a volume of 2 ml kg^{-1}, and in mice in a volume of 10 ml kg^{-1}. Experiments were performed by an observer blind to the treatment. All experimental procedures were approved by Animal Care and Use Committee at the Institute of Pharmacology, Polish Academy of Sciences in Kraków.

Drugs

2-Methyl-6-(phenylethynyl)-pyridine (MPEP) was synthesized as described previously (Gasparini *et al.*, 1999). MPEP and diazepam (Polfa-Poznań, Poland) were suspended in a 1% aqueous solution of Tween 80. Imipramine hydrochloride (Polfa-Starogard Gdański, Poland) and L-5-hydroxytryptophan (L-5-HTP; Sigma, St. Louis, MO, U.S.A.) were dissolved in sterile saline. MPEP was administered intraperitoneally (i.p.) or perorally (p.o.), diazepam, imipramine and L-5-HTP were administered i.p. All compounds were given at 60 min before the tests.

Conflict drinking test (Vogel test)

A modification of the method of (Vogel *et al.*, 1971) described below was used. On the first day of the experiment, the rats were adapted to the test chamber for 10 min. It was a plexiglass box (27 × 27 × 50 cm), equipped with a grid floor of stainless steel bars and a drinking bottle containing tap water. After the adaptation period, the animals were deprived of water for 24 h, and were then placed in the test chamber for another 10 min adaptation period, during which they had a free access to the drinking bottle. Afterwards, they were allowed a 30 min free-drinking session in their home cage. After another 24 h water deprivation period, the rats were again placed in the test chamber and were allowed to drink for 30 s. Immediately afterwards, drinking attempts were punished with an electric shock (0.5 mA). The impulses were released every 2 s (timed from the moment when a preceding shock was delivered), between the grid floor and the spout of the drinking bottle. Each shock lasted for 1 s and if the rat was drinking when an impulse was released, it received a shock. The number of shocks accepted throughout a 5 min experimental session was recorded. MPEP (0.3, 1 and 10 mg kg^{-1}, i.p.) and diazepam (10 mg kg^{-1}, i.p.) were administered 60 min before the test.

Shock threshold and free-drinking tests

To control the possibility of drug-induced changes in the perception of a stimulus or in the thirst drive, which might have contributed to the activity in the conflict drinking test, stimulus threshold measurements and a free-drinking experiment were also carried out. In both cases, the rats were treated before the experiment in the same manner as described in the conflict drinking test, including two 24 h water deprivation periods separated by 30 min of water availability. In the shock threshold test, the rats were placed individually in the box, and electric shocks were delivered through the grid floor. The shock threshold was determined stepwise with 15 s shock free intervals by manually increasing the current (0.1, 0.2, 0.3, 0.4, 0.5 mA). The shock lasted for 1 s and was delivered through the grid-floor until a rat showed an avoidance reaction (jump or jerk) to the electric stimulus.

In the free-drinking test, each animal was allowed to drink from the water spout. Licking was not punished. The total amount of water (ml), consumed in 5 min, was recorded for each rat. MPEP (1 and 10 mg kg^{-1}, i.p.) was administered 60 min before the test.

Water intake test

The rats were housed and tested in individual cages (40 × 27 × 15 cm), with free access to food and water at all times. On the day of the test, water bottles were weighed at the time of drug administration. Water was presented immediately after drug injection. Water intake (ml) was recorded at 1, 2, 4, 6 and 24 h time points. L-5-hydroxytryptophan (L-5-HTP) was used as a reference drug (Rowland *et al.*, 1987). MPEP (1 and 10 mg kg^{-1}, i.p.) and L-5-HTP (20 mg kg^{-1}, i.p.) were administered immediately before the test.

Elevated plus-maze test

The construction and the testing procedure of the elevated plus-maze were based on a method described by Pellow & File (1986). Each rat was placed in the centre of the plus-maze, facing one of the enclosed arms immediately after a 5 min adaptation in a wooden box (60 × 60 × 35 cm). During a 5 min test period, two experimenters, who were sitting in the same room approximately 1 m from the end of the open arms, recorded the number of entries into the closed or the open arm, as well as the time spent in each type of arms. The entry with all four feet put onto one arm was defined as an arm entry. At the end of each trial the maze was wiped clean. MPEP (1, 3 and 10 mg kg^{-1}, i.p. or 10 and 30 mg kg^{-1}, p.o.) and diazepam (1.25, 2.5 and 5 mg kg^{-1}, i.p.) were administered 60 min before the test.

Four-plate test

The box is made of an opaque plastic and has the shape of a rectangle (25 × 18 × 16 cm). The floor is covered with four

rectangular metal plates (11.3 x 7.7 cm) separated by a gap of 4 mm. The plates are connected to a source of direct current and the 180 V difference of potential between two adjacent plates occurs for 0.5 s when the experimenter presses a switch. Single mice were placed gently onto the plate, and allowed to explore for 15 s. Afterwards, each time a mouse passed from one plate to another, the experimenter electrified the whole floor, which evoked a visible flight reaction of the animal. If the animal continued running, it received no new shocks for the following 3 s. The number of punished crossings was counted for 60 s (Aron *et al.*, 1971). MPEP (3, 10 and 30 mg kg^{-1}, i.p.) and diazepam (2 mg kg^{-1}, i.p) were administered 60 min before the test.

Rota-rod test

Mice were preselected 1 h before the test on the rotating rod (3 cm in diameter, 6 r.p.m). Those staying on the rotating road for 2 min (approximately 95% of animals) were placed again on the same rotating rod after drug administration and were observed for 2 min. The number of animals falling from the rota-rod within 2 min was recorded. MPEP (30 mg kg^{-1}, i.p.) was administered 60 min before the test.

Open field test

The studies were carried out with rats according to a slightly modified method of Janssen *et al.* (1960). The centre of the open arena (1 m in diameter), divided into six symmetrical sectors without walls, was illuminated with a 75 W electric bulb hung directly 75 cm above it. During all the experiments the laboratory room was dark. Individual control or drug-injected animals were placed gently in the centre of the arena and were allowed to explore freely. The time of walking, ambulation (the number of crossing of sector lines) and the number of rearing and peeping episodes (looking under the edge of the arena) were recorded for 3 min. MPEP (3 and 10 mg kg^{-1}, i.p.) was administered 60 min before the test.

Behavioural despair test

The studies were carried out on rats according to the method of Porsolt *et al.* (1978). Briefly, the rats were placed individually into a glass cylinder (height 40 cm; diameter 18 cm) containing 15 cm of water, maintained at 25°C. After 15 min they were removed to a drying room (30°C) for 30 min. They were replaced in the cylinder 24 h later and the total duration of immobility was measured during a 5 min test. MPEP (0.1, 1 and 10 mg kg^{-1}, i.p.) and imipramine (30 mg kg^{-1}, i.p.) were administered 60 min before the test.

Tail suspension test

Immobility was induced by tail suspension according to the procedure of Steru *et al.* (1985). C57BL/6J mice were hung individually on a plastic string, 75 cm above the table top with an adhesive tape placed *ca.* 1 cm from the tip of the tail. Duration of immobility was recorded for 8 min. Mice were considered immobile only when they hung passively and completely motionless. MPEP (0.1, 1, 10 and 20 mg kg^{-1}, i.p.) and imipramine (20 mg kg^{-1}, i.p.) were administered 60 min before the test.

Analysis of the data

The data obtained were presented as means ± s.e.mean and evaluated using one-way ANOVA, followed by Dunnett's *post hoc* determination, using GraphPad Prism version 3.00 for Windows 97 (Graph Pad Software, San Diego CA, U.S.A.).

Results

Conflict drinking test in rats

MPEP, which at a dose of 0.3 mg kg^{-1} was not effective, at doses of 1 and 10 mg kg^{-1} i.p. significantly ($F(3,30) = 11.193$, $P < 0.001$), increased the number of shocks (by 330 and 507%, respectively) accepted during the experimental session in the Vogel test (Figure 1). The maximal effect of MPEP at a dose of 10 mg kg^{-1} was comparable to that seen with diazepam at a dose of 10 mg kg^{-1}. At the effective doses in the conflict drinking test, neither the threshold current (0.4 ± 0.04 mA) nor the water intake (10.6 ± 0.6 ml) were changed compared to vehicle treatment (Table 1). As a control water intake in non-deprived rats was also evaluated. MPEP tested at doses effective in the conflict drinking test (1 or 10 mg kg^{-1}) had no significant effect on water consumption, while L-5-HTP (20 mg kg^{-1}) used as a standard drug (Rowland *et al.*, 1987) significantly increased the water intake (Table 2).

Plus-maze test in rats

The total number of entries (open + closed arm entries) observed with control rats during the 5 min test session was about six in the present set of experiments and was taken as 100%. In control rats 32.7, 34,4 and 38.5% of the entries were made into the open arms (Table 3), and 8.7, 9.0 and 10.9% of the total time (255 s) spent in the arms (either type) was spent in the open arms. MPEP administered at a dose of 1 mg kg^{-1} i.p. did not change the entries into and time spent

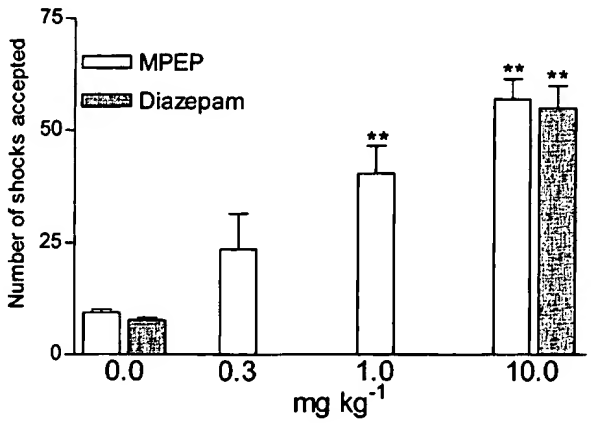

Figure 1 Effects of MPEP and diazepam in the conflict drinking test in rats. MPEP and diazepam were administered i.p. at 60 min before the test. The given values represent the mean ± s.e.mean of the number of shocks accepted during a 5 min experimental session, $n = 7-9$, ** $P < 0.01$ vs control group.

Table 1 The effect of MPEP on the shock threshold and the amount of water consumed in water deprived rats

Compound	*Dose* mg kg^{-1}	*Shock threshold* (mA)	*Water consumption* (ml)
Vehicle	–	0.4±0.04	10.6±0.6
MPEP	1	0.4±0.03	11.4±0.3
	10	0.3±0.01	9.9±0.6

MPEP was administered i.p. 60 min before tests. Values are the means±s.e.mean, $n=7$.

Table 2 Effects of MPEP and L-5-HTP on the amount of water intake in water non-deprivated rats

Compound	*Dose* mg kg^{-1}	*Water consumption* (ml) 1 h	2 h	4 h	6 h	24 h
Vehicle	–	1.0±0.4	2.1±0.5	2.4±0.6	2.7±0.6	35.4±2.1
MPEP	1	0.8±0.5	1.4±0.4	1.6±0.6	1.8±0.6	32.3±1.1
MPEP	10	0.6±0.1	1.5±0.5	1.7±0.5	1.8±0.6	33.1±2.7
L-5-HTP	20	2.8±0.8*	3.8±0.8*	4.0±0.9*	4.1±0.9*	39.4±3.3

MPEP and L-5-HTP were administered i.p. immediately before the test. The values are the means±s.e.mean, $n=6$. *$P<0.05$ vs vehicle group.

Table 3 The effects of MPEP and diazepam in the plus-maze test in rats

Compound	*Dose* mg kg^{-1}	*% of time in open arms*	*% of open arm entries*
Vehicle	–	8.7±0.5	32.7±5.2
MPEP	1 i.p.	16.2±5.8	38.7±8.2
	3 i.p.	45.1±7.1**	48.3±2.2
	10 i.p.	73.8±8.3**	67.7±6.7**
Vehicle	–	9.0±1.9	34.4±3.9
MPEP	10 p.o.	15.0±2.4	29.1±4.4
	30 p.o.	63.7±12.3**	62.6±9.2*
Vehicle	–	10.9±1.1	38.5±3.1
Diazepam	1.25 i.p.	20.3±5.5	45.2±8.9
	2.5 i.p	47.2±5.3*	73.8±4.2*
	5 i.p.	70.4±10.9**	76.2±8.8**

MPEP was administered i.p. or p.o. and diazepam i.p. 60 min before the test. Values are the means±s.e.mean, $n=6-7$, *$P<0.05$, **$P<0.01$ vs respective vehicle group.

in the open arms. When given at doses of 3 and 10 mg kg^{-1} i.p. it significantly (F (3,24)=22.978, $P<0.001$) dose-dependently increased the time spent in the open arms (up to 45 and 74%, respectively), and the percentage of entries into the open arms (up to 48 and 68%, respectively, F (3,24)=5.678, $P<0.01$) (Table 3). MPEP at doses of 3 and 10 mg kg^{-1} i.p. significantly increased (by 64%) the total number of entries and reduced (by about 25%) the total time spent (data not shown) in the arms (either type). After p.o. administration higher doses of MPEP were required to induce significant behavioural changes: at the dose of 30 mg kg^{-1} (but not 10 mg kg^{-1}) MPEP significantly (up to 64%, F (2,16)=14.249, $P<0.001$) increased the percentage of the time spent in the open arms and the percentage of entries into the open arms (up to 63%, F (2,16)=7.295, $P<0.01$). MPEP given p.o. in both doses used did not change the total number of entries nor the total time spent in the arms (either type). Diazepam, i.e. the positive standard, administered in a dose of 1.25 mg kg^{-1} i.p. was ineffective in that test, however when given at doses of 2.5 and 5 mg kg^{-1} i.p. it significantly (F (3,22)=14.52, $P<0.001$) increased the percentage of the time spent in the open arms (up to 47 and 70%, respectively), as well as the percentage of entries into the open arms (up to 74 and 76%, respectively (F (3,22)=5.871, $P<0.01$) (Table 3). Diazepam at a dose of 5 mg kg^{-1} (but not lower) significantly reduced (by 52%) the total number of entries (data not shown).

Open field test in rats and rota-rod test in mice

MPEP at doses of 3 and 10 mg kg^{-1} i.p. did not change exploratory locomotor activity in rats (F (2,18)=2; 0.273; 0.011, n.s.), as evaluated by the open field test (Table 4). MPEP at a dose of 30 mg kg^{-1} i.p. did not disturb endurance performance on the rotating rod in mice (data not shown).

Four-plate test in mice

MPEP administered at 30 mg kg^{-1} i.p. slightly but significantly increased (by 39%) the number of punished crossings in the four-plate test (Figure 2), lower doses of the compound (3 and 10 mg kg^{-1}) did not affect the number of punished crossings in that test (F (3,36)=3.240, $P<0.05$). Diazepam, i.e. the positive standard, in a dose of 2 mg kg^{-1} increased the number of crossings by 70%.

Table 4 The effect of MPEP on the exploratory activity in the open field test in rats

Compound	Dose mg kg^{-1}	Exploratory activity: Time of walking (s)	Exploratory activity: Ambulation	Exploratory activity: Peeping + rearing
Vehicle	–	44.9±2.7	14.6±1.4	9.9±1.2
MPEP	3	44.3±1.7	15.1±0.5	10.4±0.8
	10	44.9±4.3	18.3±2.0	11.1±1.5

MPEP was administered i.p. 60 min before the test. Values are means±s.e.mean, $n=6$.

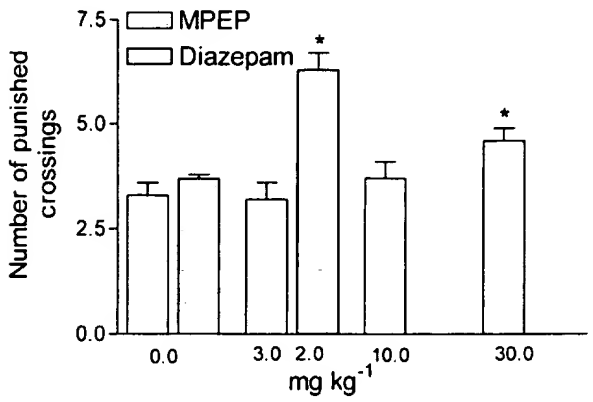

Figure 2 Effects of MPEP and diazepam in the four-plate test in mice. MPEP and diazepam were administered i.p. 60 min before the test. The given values represent the mean±s.e.mean of the number of shocks accepted during a 1 min experimental session, $n=10$. *$P<0.05$ vs control group.

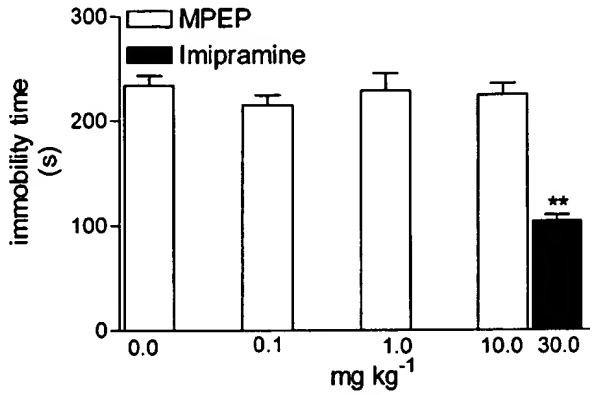

Figure 3 The effects of MPEP and imipramine on the total duration of immobility in the forced swimming test in rats. MPEP and imipramine were administered i.p. at 60 min before the test. Values represent the mean±s.e.mean of the immobility time during a 5 min experimental session, $n=9-10$. ** $P<0.01$ vs control group.

Behavioural despair test in rats and tail suspension test in mice

MPEP in doses of 0.1, 1 and 10 mg kg^{-1} i.p. did not change the behaviour of rats in the behavioural despair test, while imipramine, 30 mg kg^{-1}, used as standard drug, significantly ($F(6,49)=25.02$, $P<0.001$) decreased the immobility time in that test (Figure 3).

MPEP used in doses of 1, 10 and 20 mg kg^{-1} significantly (by 55% after the highest dose), ($F(3,28)=15.47$, $P<0.001$) decreased the immobility time of mice in the tail suspension test. Its efficacy was similar to that of imipramine (20 mg kg^{-1}), used as the positive standard (Figure 4).

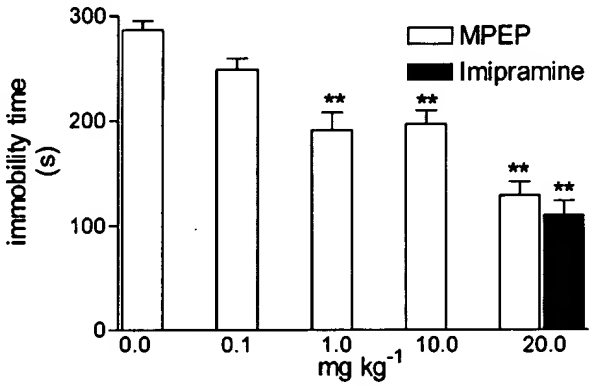

Figure 4 The effects of MPEP and imipramine on the total duration of immobility in the tail suspension test in mice. MPEP and imipramine were administered i.p. at 60 min before the test. Values represent the mean±s.e.mean of the immobility time during a 8 min experimental session, $n=9-10$. ** $P<0.01$ vs control group.

Discussion

Anxiolytic-like effects of MPEP

Benzodiazepines which are the most commonly used anxiolytic drugs, act *via* facilitation of the inhibitory GABA-ergic transmission. Benzodiazepines are effective agents, but disadvantageous side effects such as sedation, ataxia and abuse liability are associated with their administration. Decreased glutamatergic transmission, which leads to overall inhibitory effects in the central nervous system may have consequences similar to the effect of increased GABA-ergic transmission. Hence substances which inhibit stimulatory glutamatergic neurotransmission may possess anxiolytic effects. Indeed, antagonists of ionotropic glutamate receptors exhibit an anxiolytic-like activity in animal models (Stephens *et al.*, 1986; Bennett *et al.*, 1989; Jessa *et al.*, 1996), however the potential clinical utility of competitive and noncompetitive NMDA antagonists is strictly limited by their undesirable side effects (Danysz & Parsons, 1998).

Substances which influence mGlu receptors, including agonists of group II mGluR and antagonists of group I mGluR, can also exert an inhibitory function in the brain

(Conn & Pin 1997). Our earlier data have shown that (S)-4-carboxy-3-hydroxyphenylglycine (S-4C3H-PG), an antagonist of group I mGluR, exhibits anxiolytic-like activity in animals (Chojnacka-Wójcik *et al.*, 1997). However, S-4C3H-PG is also an agonist of group II mGluR (Sekiyama *et al.*, 1996) and agonists of group II mGluR exert anxiolytic-like effects in animals (Helton *et al.*, 1998; Kłodzińska *et al.*, 1999).

In order to further investigate the involvement of group I mGlu receptors in anxiety, we decided to evaluate the action of the selective antagonist of the mGlu5 receptor MPEP, which is devoid of agonist activity on group II mGlu receptors and which penetrates into the brain (Gasparini *et al.*, 1999). An anxiolytic-like effect of MPEP was evaluated in three behavioural tests: the rat Vogel test (Vogel *et al.*, 1971), the elevated plus-maze test (Pellow & File, 1986), and the four-plate test in mice (Aron *et al.*, 1971). In the elevated plus-maze test the total number of entries (open + closed arm entries/test session) is taken as an index of drug effect on the locomotor activity, but this is a relatively insensitive measure (Dawson & Tricklebank, 1995). MPEP caused a small but significant increase in the total number of entries into the arms of the maze, but did not change the exploratory activity of rats in the open field test. Therefore, the increase in the percentage of the open arm entries/time spent on the open arms induced by MPEP is likely to reflect a specific anti-anxiety effect and can not be explained by competing behaviour such as exploration. This is further supported by the anxiolytic-like effects of MPEP in two conditioned response paradigms, i.e. the Vogel test and the four-plate test. In the Vogel test in rats the action of MPEP was not related to reduced perception of the stimulus or to an increased thirst drive. Preliminary findings of anxiolytic-like effects of MPEP in unconditioned response tests (social exploration test, stress-induced hyperthermia and marble burying test (Spooren *et al.*, 2000), suggest, that MPEP exhibits anxiolytic effects in various rodent models of anxiety. Taken together, all the data suggests that MPEP produces potential anti-anxiety effects and indicate an involvement of mGlu5 receptors in anxiety.

The hippocampus is involved in anxiety (Gray, 1982) and effects of different anxiolytics, including a variety of agents acting upon the glutamatergic system (e.g. Przegalinski *et al.*, 1997). In the hippocampus, a high expression of mRNA for group I mGlu receptors (see Testa *et al.*, 1998), as well as the high immunoreactivity of group I mGlu receptors (Shigemoto *et al.*, 1997; Blumcke *et al.*, 1996) were found. That structure is also intensely immunolabelled by mGluR5 antibody (Lujan *et al.*, 1996). The ability of S-4C3H-PG, an antagonist of group I mGlu receptors to produce anxiolytic responses in the Vogel test after intrahippocampal administration (Chojnacka-Wójcik *et al.*, 1997), further suggests that this structure might be related to anxiolytic effects of group I mGlu receptor antagonists including MPEP. To verify that hypothesis experiments with intra-hippocampal injections of the MPEP are in progress.

Antidepressant-like effects of MPEP

The antidepressant-like effects of MPEP were evaluated in two behavioural tests, the tail suspension test in mice and the Porsolt test in rats. While MPEP did shorten the immobility time in one model of depression in mice, a tail suspension test, it was inactive in the behavioural despair test. The tail suspension test shows a higher predictive validity for identifying potentially useful pharmacotherapies for depression, compared to the behavioural despair test, e.g. it detects the antidepressant effects of specific serotonin reuptake inhibitors (Ali-Kodja *et al.*, 1986), and as shown by the above results, an action of the mGlu5 receptor antagonist. Earlier data indicate that the excitatory effect of an agonist of the group I mGlu receptor system is influenced by prolonged treatment with an antidepressant drug imipramine or by chronic electroconvulsive (ECS) treatment (Palucha *et al.*, 1997; Pilc *et al.*, 1998). In those experiments performed in the CA1 area of the hippocampus, the (R,S)-3,5-dihydroxyphenylglycine-mediated increase of the population spike, was attenuated both by chronic imipramine and ECS (Palucha *et al.*, 1997; Pilc *et al.*, 1998). It can be speculated therefore, that it is the inhibition of group I mGlu receptor mediated neurotransmission which can contribute to the antidepressant-like effects of both MPEP and imipramine or ECS.

The preclinical data indicate that compounds which reduce transmission at NMDA receptors behave like antidepressants (Skolnick, 1999). Glutamatergic transmission *via* stimulation of group I mGlu receptors has also been shown to potentiate the ionotropic glutamate responses in various preparations (Glaum & Miller, 1994), including potentiation of NMDA currents (Fitzjohn *et al.*, 1996; Ugolini *et al.*, 1999). The blockade of group I mGlu receptors by MPEP may therefore lead to a decrease in NMDA-receptor-mediated neurotransmission and might contribute to the antidepressant-like effect of MPEP. It can be speculated that MPEP, which neither causes sedation nor disturbs the rota-rod performance, might be free of side effects produced by antagonists of NMDA receptors.

In conclusion, MPEP is a selective, systematically active antagonist of mGlu5 receptors. It produced anxiolytic-like effects in several tests such as the Vogel test in rats, the elevated plus-maze test in rats as well as the four-plate test in mice. MPEP also exerted antidepressant-like effects in the tail suspension test in mice. It was also found that MPEP did not induce sedation nor disturb motor coordination in animals. The above results indicate that antagonists of mGlu5 receptors may play a role in the therapy of anxiety and/or depression. Identification of the sites of action of MPEP and of the mechanism of these effects still requires further studies.

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The metabotropic glutamate receptor antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP) blocks fear conditioning in rats

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Abstract

Glutamate receptors play an essential role in fear-related learning and memory. The present study was designed to assess the role of the group I metabotropic glutamate receptor (mGluR) subtype 5 in the acquisition and retrieval of conditioned fear in rats. The selective mGluR5 antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP) was applied systemically (0.0, 0.3, 3.0, 30.0 mg/kg per os) 60 min before the acquisition training and before the expression of conditioned fear, respectively, in the fear-potentiated startle paradigm. MPEP dose-dependently blocked the acquisition of fear. This effect was not due to state-dependent learning. MPEP also prevented the expression of fear at a dose of 30.0 mg/kg. As a positive control for these effects, we showed that the benzodiazepine anxiolytic compound diazepam (1.25 mg/kg intraperitoneally) also blocked acquisition and expression of fear potentiated startle. MPEP did not affect the baseline startle magnitude, short-term habituation of startle, sensitisation of startle by footshocks or prepulse inhibition of startle. These data indicate a crucial role for mGluR5 in the regulation of fear conditioning. In the highest dose MPEP might exert anxiolytic properties.

Keywords: Acoustic startle response; Fear; Learning; Memory; Metabotropic glutamate receptors; Prepulse inhibition

1. Introduction

Fear conditioning is a simple form of Pavlovian conditioning that serves as a model for learning and memory (Fendt and Fanselow, 1999; LeDoux, 1992). The mechanisms underlying the acquisition and retrieval of conditioned fear in rodents are also relevant for the understanding of human fear and anxiety, and possibly for the design of new anxiolytic compounds (Davis et al., 1993). In Pavlovian fear conditioning paradigms, a neutral stimulus is paired with an aversive event and comes to predict this aversive event even after very few pairings. After training the conditioned stimulus elicits or potentiates protective responses such as the acoustic startle response (ASR), freezing, vocalisation or an increase in arterial blood pressure (LeDoux, 1995).

Glutamate receptors play an essential role in learning and memory and, therefore, are also involved in fear conditioning. Both types of ionotropic glutamate receptors contribute to conditioned fear. For example, it has been shown that the acquisition of fear in the fear-potentiated startle paradigm can be blocked by intra-amygdaloid infusion of the NMDA receptor antagonist AP-5 (Miserendino et al., 1990; Gewirtz and Davis, 1997). The retrieval of fear memories in this paradigm was blocked by intra-amygdaloid infusion of the AMPA/Kainate receptor antagonist CNQX (Kim et al., 1993). The contribution of mGluRs to fear conditioning is less well investigated although it is known that mGluRs are involved in learning (Riedel, 1996). Group I mGluR antagonists blocked memory consolidation of contextual conditioning (Nielsen et al., 1997) and fear-conditioning led to a transient up-regulation of mGluR5 in the hippocampus (Riedel et al., 2000). A previous

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study has shown that the selective group II mGluR antagonist LY354740 exerts anxiolytic properties in the fear-potentiated startle and elevated plus maze paradigms (Helton et al., 1998).

The mGluRs are interesting targets for the pharmacological treatment of neurological and/or psychiatric disorders that are based on a dysfunction of the glutamatergic system. Molecular biology studies revealed that mGluRs can be subdivided into eight distinct subtypes that belong to three different groups (groups I–III), based upon their sequence homology, pharmacology and intracellular second-messenger systems (Conn and Pin, 1997; Anwyl, 1999).

MPEP is a highly potent mGluR5 antagonist with minimal activity at other metabotropic or ionotropic glutamate receptors (Gasparini et al., 1999). In order to assess the potential role of mGluR5 in fear conditioning we tested whether MPEP affects the acquisition and/or retrieval of fear in the fear-potentiated startle paradigm. As a positive control (Davis, 1979), we also measured the effect of the clinically prescribed anxiolytic diazepam on fear-conditioning. In addition, we tested the effects of MPEP on habituation and prepulse inhibition of the ASR, as well as on sensitisation of the ASR by footshocks.

2. Methods

2.1. Animals and behavioural procedures

A total of 225 experimentally naive male Sprague–Dawley rats (Charles River, Sulzfeld, Germany) weighing between 210 and 300 g were used in this study. The animals were housed in groups of 5–6 in standard Macrolon cages under a constant light/dark cycle (7 a.m. to 7 p.m. light on) and received food and water ad libitum. After arrival, they were handled daily and accustomed to the oral gavage needle used for the peroral drug application precedure. Food was removed 2 h before drug administration. The experiments were done in accordance with the NIH ethical guidelines for the care and use of laboratory animals for experiments and were approved by the local animal care committee (Regierungspräsidium Tübingen, ZP 3/99).

2.2. Fear-conditioning

One hundred rats were trained in two identical, acoustically isolated dark boxes (38×60×28 cm^3). The floor of the box was composed of stainless steel bars spaced 15 mm apart, through which the unconditioned stimulus (US), a 0.6 mA footshock with 0.5 s duration was administered. The footshocks were produced by a shock generator (custom-made at the University of Tübingen) located outside the conditioning box. The conditioned stimulus (CS) for fear-conditioning was a 3.7 s white light produced by a 15 W bulb. The light bulb was located at the top of the boxes. Stimulus presentations were controlled by a computer and an digital–analogue-interface (Hortmann universal function synthesiser, Neckartenzlingen, Germany). The animals were randomly assigned to the different treatment groups. Forty rats received MPEP (0.0, 0.3, 3.0, 30.0 mg/kg) 60 min before training (acquisition of fear), 42 rats received MPEP before the startle test (expression of fear). In order to control for a possible state-dependency of the acquisition of fear after MPEP-treatment, 18 rats received vehicle, 3.0 mg/kg MPEP or 30.0 mg/kg MPEP (N=6 in each group) both before training and before testing. Thirty rats received diazepam (0.0, 1.25 mg/kg) before fear conditioning or startle testing.

2.3. Fear-potentiation of the acoustic startle response (ASR)

On the first day, the rats were placed into the training boxes for fear-conditioning and after an acclimatisation time of 5 min, they were trained to associate the light with the footshock. Ten pairings of the light with 0.6 mA footshock were given. The US was presented during the last 0.5 s of the 3.7 s light CS with a mean intertrial interval of 2 min (range 1.5–2.5 min).

Two days later, the rats were tested for fear-potentiation of the ASR. They were placed into the test chamber and after an acclimatisation time of 5 min 10 initial startle stimuli (100 dB SPL, 10 kHz tone pulse, 20 ms duration including 0.4 ms rise and fall times, 30 s interstimulus interval) were presented in order to induce a stable baseline of ASR magnitude. Then, each rat received 20 startle stimuli, one half presented 3.2 s after the onset of the light CS, the other half presented in the absence of the light (tone-alone trials). All trial types were presented in a pseudo-randomised order (30 s interstimulus interval). The difference between the tone-alone and the light–tone trials is an operational measure of fear (Davis et al., 1993; Fendt and Fanselow, 1999; Koch, 1999). The ASR and fear-potentiated startle were measured in 2 identical sound-attenuated test chambers (100×80×60 cm^3). The rats were placed in wire mesh test cages (20×10×12 cm^3) with a steel floor, mounted on a piezoelectric accelerometer (custom-made at the University of Tübingen). Movements of the rats resulted in changes of the voltage output of the accelerometers. These signals were amplified, digitised and transmitted via an analogue–digital-interface into a computer for further analysis. A loudspeaker, set up at a distance of 40 cm from the wire mesh cage, delivered the acoustic startle stimuli and a continuous white background noise (52 dB SPL). The presentation of the acoustic stimuli was controlled by a computer and a DA-interface (Hortmann universal function synthesiser, Hortmann,

Neckartenzlingen, Germany). The whole body startle amplitude was calculated from the difference between the peak-to-peak voltage output of the accelerometer within time-windows of 80 ms after and 80 ms before the startle stimulus onset. The spontaneous motor activity was calculated as the root mean square (RMS) value of the accelerometer output, measured in a time window of 28 s between the different test trials. All data are arbitrary units of the accelerometer output.

2.4. Habituation

The effect of MPEP on habituation of the ASR was also assessed in the test-session for fear-potentiation of the ASR. Short-term habituation is a measure of non-associative learning. The decline of the ASR magnitude across the initial 10 startle stimuli was taken as the measure of short-term habituation of the ASR. The mean ASR magnitudes of 2 trials were lumped together and the differences between the first block and the last block of 2 trials was compared between the different treatment groups.

2.5. Sensitisation

For the assessment of the effect of MPEP on the perception of footshocks, the sensitisation of the ASR by footshocks was tested in 16 rats as described before (Fendt et al., 1994). Briefly, the rats were placed in a test cage provided with a footshock-grid. After 5 min acclimatisation, they received 40 startle stimuli (as above), then 10 footshocks (0.6 mA, 0.5 s duration, 1 Hz) were given through the shock-grid, then a further 40 startle stimuli were presented. Sensitisation was assessed by comparing the mean ASR magnitude of 20 trials before the footshocks with the ASR magnitude of 30 trials after the presentation of footshocks. However, the 5 trials immediately after shock presentation were not included in these 30 trials, as described before (Davis, 1989; Fendt et al., 1994). The accelerometer output during presentation of the footshocks was taken as a measure of the rats immediate motor response to the footshocks.

2.6. Prepulse inhibition (PPI)

Prepulse inhibition (PPI) of the ASR was assessed in order to provide a measure of sensorimotor gating. Seventy nine rats were divided into four treatment groups (0.0, 0.3, 3.0, 30.0 mg MPEP/kg). Sixty min after drug administration, they were placed in the test cages, and after a 5 min acclimatisation period the test session began with an initial startle stimulus followed by 4 different trial types presented in a pseudorandom order: 1, Pulse alone (100 dB SPL broad band noise pulse, 20 ms duration); 2, Prepulse (60 dB SPL, 10 kHz tone pulse, 20 ms duration, including 0.4 ms rise/fall times) followed by a pulse 100 ms after prepulse-onset; 3, 60 dB-prepulse alone; and 4, No stimulus. Background noise intensity was 52 dB SPL. A total of 5 presentations of each trial type was given with an interstimulus interval of 30 s. PPI was measured as the difference between the pulse alone trials and the prepulse–pulse trials and expressed as percent PPI [100×(mean ASR amplitude on pulse alone trials−mean ASR amplitude on prepulse−pulse trials)/mean ASR amplitude on pulse alone trials]. The response to the single pulse at the beginning of the test session was discarded. We here used a relatively weak prepulse in order to avoid possible floor or ceiling effects of PPI performance.

2.7. Drugs

MPEP (0.0, 0.3, 3.0, 30.0 mg/kg) was freshly dissolved in 0.5% methyl cellulose (Sigma, Deisenhofen, Germany) and administered per os through a bulb-tipped oral gavage needle 60 min before the beginning of the conditioning or of the tests. Diazepam (0.0, 1.25 mg/kg; Valium® MM Roche, Hoffmann–La Roche AG, Grenzach-Wyhlen, Germany) was dissolved in saline and administered intraperitoneally 30 min before conditioning or testing.

2.8. Data analysis

All data evaluations were done with one-way analyses of variance (ANOVA) followed by post-hoc Tukey's *t*-tests, or with Student's *t*-tests.

3. Results

3.1. Effect of MPEP on acquisition and expression of fear conditioning

Fig. 1 illustrates the effect of MPEP on fear-potentiated startle. MPEP administered before fear conditioning dose-dependently blocks the acquisition of fear ($F_{3,36}$=3.24; p=0.033). No effect on the baseline ASR magnitude (tone-alone trials) was found ($F_{3,36}$=0.46; p=0.716). 3 mg/kg MPEP also blocked fear-potentiated startle if administered both before training and before testing ($F_{2,15}$=3.86; p=0.044) (Fig. 2). Administration of MPEP after conditioning, 1 h before startle testing blocks fear-potentiation only at the highest dose ($F_{3,37}$=4.67; p=0.007). No effect of MPEP on baseline ASR magnitude (0.0 mg/kg: 111±23; 0.3 mg/kg: 104±18; 3.0 mg/kg: 98±23; 30 mg/kg: 101±13. $F_{3,37}$=0.084; p=0.968) and spontaneous motor activity (RMS values) of the rats in the test cage (0.0 mg/kg: 163±40; 0.3 mg/kg: 138±25; 3.0 mg/kg: 155±11; 30 mg/kg: 176±31. $F_{3,37}$=0.376; p=0.771) was found. 3

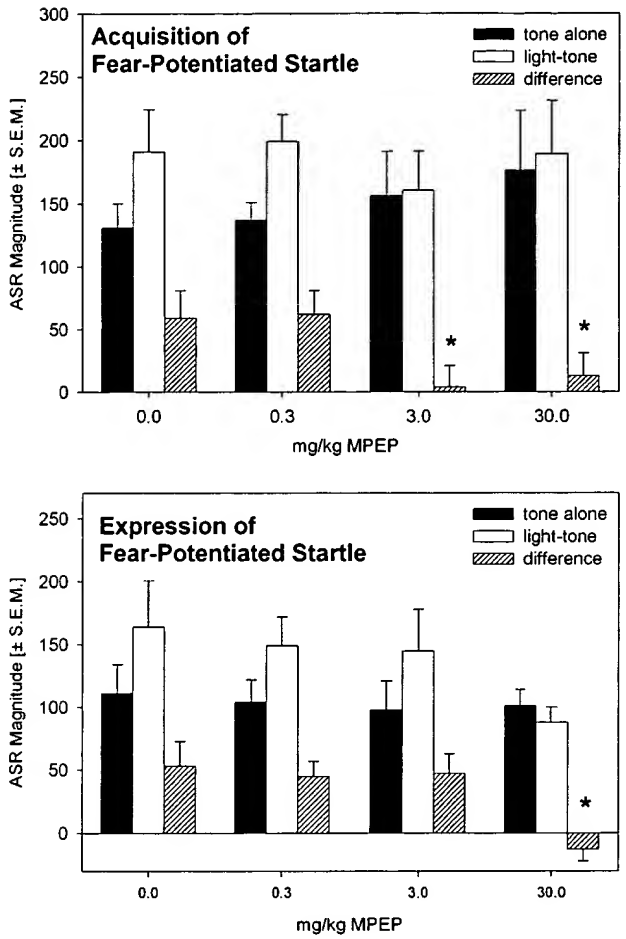

Fig. 1. Effects of MPEP on fear-potentiation of the ASR. Bar diagrams show the mean ASR magnitude in the absence (black bars) or presence (white bars) of the light-CS. Hatched bars represent the difference between tone-alone and light–tone trials which is taken as an index of fear. MPEP dose-dependently reduced the acquisition of fear if administered 60 min before training (upper panel). MPEP also blocked the retrieval (expression) of fear if administered 60 min before the test (bottom panel). Asterisks indicate $p<0.05$ computed by an ANOVA followed by post-hoc Tukey's t-test for pairwise comparisons with the vehicle-group.

mg/kg MPEP also blocked fear-potentiated startle if administered both before training and before testing ($F_{2,15}$=3.86; p=0.044) (Fig. 2).

Diazepam significantly blocked both acquisition and expression of fear in the fear-potentiated startle paradigm (% difference in arbitrary units between tone alone and light–tone trials, n=10 in each group: 0.0 mg/kg: 46±21 for expression of fear; 1.25 mg/kg: 13±10 for expression of fear; 1.25 mg/kg: 24±10 for acquisition of fear. $F_{2,25}$=4.916; p=0.016); a trend of decrease of the baseline ASR magnitude (tone-alone trials) 30 min after administration of diazepam was observed (0.0 mg/kg: 93±16; 1.25 mg/kg: 80±18. $F_{2,25}$=2.635; p=0.092).

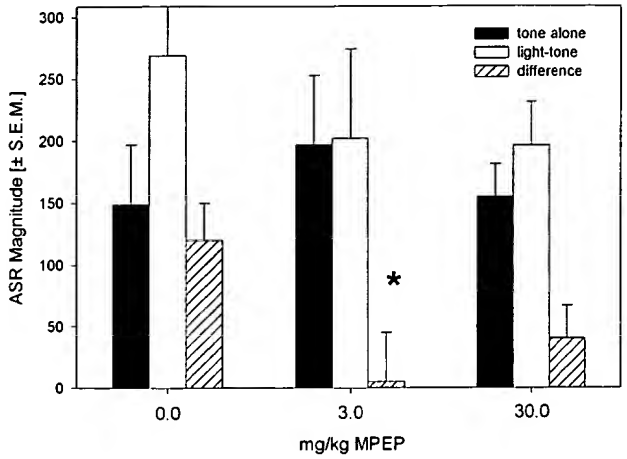

Fig. 2. Effects of MPEP on fear-potentiation of startle. Bar diagrams show the mean ASR magnitude in the absence (black bars) or presence (white bars) of the light-CS. Hatched bars represent the difference between tone-alone and light–tone trials which is taken as an index of fear. Here, MPEP was given both before conditioning and before testing to see if the blockade of fear-conditioning was due to state-dependent retrieval of fear in the test situation. 3 mg/kg MPEP significantly blocked fear-potentiation of startle suggesting no state-dependency induced by MPEP. Asterisk indicates $p<0.05$ (ANOVA followed by post-hoc Tukey's t-test).

3.2. *Effect of MPEP on sensitisation of the ASR and on shock-induced motor activity*

Fig. 3 illustrates that no significant difference between the shock-sensitisation of the ASR between vehicle-treated rats (post-shock ASR magnitude enhanced by 53%) and rats that received 3.0 mg/kg MPEP before administration of footshocks (post-shock ASR magnitude enhanced by 98%) was found (t_{14}=0.414; p=0.685). No significant difference of the accelerometer output

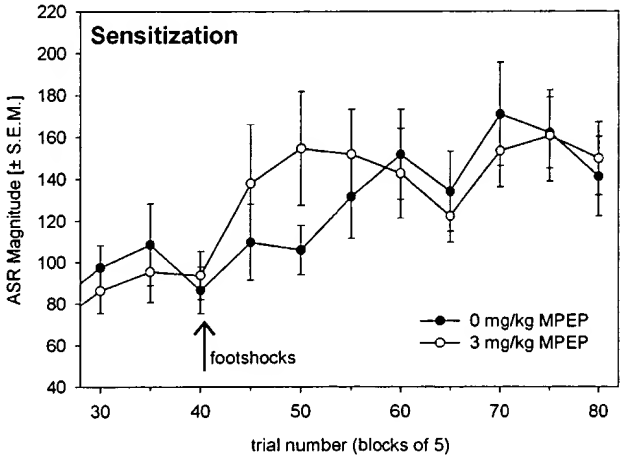

Fig. 3. No effect of 3.0 mg/kg MPEP on sensitisation of the ASR by footshocks. Both vehicle-treated rats (solid circles) and MPEP-treated rats (open circles) show an increase in mean ASR magnitude after administration of a train of 0.6 mA footshocks.

during administration of footshocks was observed between vehicle-treated and MPEP-treated rats (0.0 mg/kg: 1776±617; 3.0 mg/kg: 2265±1180. t_{14}=0.367; p=0.719).

3.3. Effects of MPEP on PPI and habituation of the ASR

MPEP did not affect PPI of the ASR (0.0 mg/kg: 26±7%; 0.3 mg/kg: 29±9%; 3.0 mg/kg: 27±9%; 30.0 mg/kg: 31±11; $F_{3,76}$=0.059; p=0.981). Similar to all the other tests, MPEP did not affect the baseline ASR magnitude in the pulse alone-trials ($F_{3,76}$=0.175; p=0.913; data not shown).

Short-term habituation of the ASR was also not affected by MPEP as indicated by the decline of the ASR magnitude across 10 startle stimuli presented before the measurement of fear-potentiated startle after vehicle-treatment and after administration of MPEP. An ANOVA computed no significant difference between the first and the last trial blocks in each group ($F_{3,38}$=0.565; p=0.641) which indicates that MPEP did not affect short-term habituation of the ASR (data not shown).

4. Discussion

The present data indicate that the subtype specific mGluR5 antagonist MPEP impairs both the acquisition and the expression of fear in the fear-potentiated startle paradigm. No effects on the baseline ASR magnitude, sensitisation by footshocks, PPI or habituation of the ASR were found.

4.1. Role of mGluR5 in the acquisition and expression of conditioned fear

Previous research revealed that mGluRs play a major role in learning (Riedel, 1996). With special reference to fear-conditioning, it was shown that knockout of the group III mGluR7 gene leads to an impairment of fear conditioning in mice possibly by a lack of autoreceptor inhibition in the amygdala (Masugi et al., 1999). However, in that study it was not clear whether the gene knockout impaired the acquisition or the expression of fear. Pharmacological studies applying group I mGluR antagonists have shown that (+)-α-methyl-4-carboxyphenylglycine (MCPG), a competitive mGluR1 antagonist with low affinity to mGluR5 and moderate affinity to group II receptors (Conn and Pin, 1997) impairs learning in the water maze but did not block the acquisition of conditioned fear (Bordi et al., 1996). A paper by Frohardt and co-workers showed that intrahippocampal infusions of MCPG reduced contextual fear, but did not affect cue-specific fear (Frohardt et al., 1999). Interestingly, fear-conditioning induced a transient overexpression of mGluR5 protein in the hippocampus suggesting an important role of these receptors in memory consolidation (Riedel et al., 2000). A recent paper has shown that the group II mGluR agonist LY354740 reduces fear in the fear-potentiated startle and the elevated plus maze paradigms without sedative or mnemonic side effects (Helton et al., 1998). Since group II mGluRs are located pre- and postsynaptically, it is conceivable that the mGluR agonist LY354740 reduces glutamatergic neurotransmission by autoreceptor activation in brain structures involved in the expression of fear, such as the amygdala (Davis et al., 1993; Fendt and Fanselow, 1999; Koch, 1999). To the best of our knowledge, the present study is the first to describe the effects of a specific mGluR5 antagonist on fear conditioning.

The finding of reduced fear conditioning after MPEP-treatment suggests that the mGluR5 antagonist impaired the association between the footshocks and the light. Since the sensitising effect of footshocks on the ASR was still observed after MPEP-treatment, and since the immediate motor response to the footshocks of the rats (jumping and flinching in the test cage) were similar in vehicle- and in MPEP-treated rats, we conclude that this is a specific learning deficit and cannot be attributed to possible analgetic or sedative effects of MPEP. It is possible that the rats which were conditioned after treatment with MPEP have acquired fear, but were unable to retrieve the fearful memory in the undrugged state. We therefore performed an additional control experiment for state-dependent learning. Since the blockade of fear-potentiated startle was also seen when the rats were tested after the same dose of MPEP that was given prior to conditioning, we conclude that the effect of MPEP on fear-potentiation of the ASR was probably not due to state-dependent learning. At least the dose of 3.0 mg/kg MPEP significantly blocked fear-conditioning when administered both before training and testing. In this control experiment for state-dependency the reduction of fear-potentiated startle was not statistically significant after administration of 30.0 mg/kg MPEP which might be interpreted as a case of state-dependent expression of fear-conditioning. However, it is also possible that at higher doses MPEP exerts non-specific effects on other mGluRs. At doses >100 μM (IC_{50}) MPEP can also affect mGluR1, 2, 4, 7, 8. Diazepam in a dose that has previously been found to reduce fear-potentiated startle (Davis, 1979; Joordens et al., 1999), also reduced fear in our conditioning and testing procedure, consistent with the amnestic and anxiolytic effect of diazepam. The fact that MPEP did not affect sensitisation of the ASR is not only important as a control for the possibility of an impairment by MPEP of footshock sensitivity. Since sensitisation has been suggested to be due to rapid context fear conditioning (Richardson and Elsayed, 1998), these findings also suggest that MPEP specifically

blocked cue-conditioning, but not the rapid context conditioning.

The dose of 3.0 mg/kg MPEP which impaired the acquisition of fear, did not affect the retrieval of fear memories if the compound was administered 60 min before the test, when the rats had learned to associate light and footshock in the absence of the drug. However, the expression of fear-potentiated startle was impaired by the highest dose of MPEP, similar to the positive control experiment using the anxiolytic compound diazepam (Davis, 1979), indicating that MPEP exerts anxiolytic properties at higher doses. The treatment with 30 mg/kg MPEP did not depress the baseline ASR magnitude (tone-alone trials). This is an important observation, because the fact that some anxiolytic drugs also reduce the ASR baseline magnitude confounds the interpretation that the drug-induced reduction of fear-potentiated startle reflects a genuine anxiolytic effect, or is simply due to motor suppression (Joordens et al., 1999). Since mGluRs have also been implicated in the consolidation of memories (Nielsen et al., 1997; Ungerer et al., 1998), we cannot exclude the possibility that MPEP administered before the test, i.e. about 48 h after training, might have interfered with memory consolidation.

It is unclear where in the brain MPEP exerted its effects on fear conditioning. A recent paper has shown that mGluR5 knockout mice show impaired learning and a disruption of hippocampal long-term potentiation (LTP) (Lu et al., 1997). However, the hippocampus is not essential for fear-potentiation of the ASR using a discrete cue (McNish et al., 1997). Conversely, it is well known that the amygdaloid complex is the key structure underlying the acquisition and expression of fear in the fear-potentiated startle paradigm (Davis et al., 1993; Koch, 1999), as well as in other paradigms (LeDoux, 1992; Fendt and Fanselow, 1999). The acquisition of fear probably occurs through processes involving LTP in the lateral/basolateral amygdala complex (McKernan and Shinnick-Gallagher, 1997; Rogan et al., 1997). Furthermore, it has been shown that the ASR can be potentiated by local intraamygdaloid application of the mGluR agonist trans-ACPD (Koch, 1993). Taken together, these data indicate that MPEP might have impaired the acquisition of fear by a blockade of mGluR5 in the amygdala. Since the expression of fear in the fear-potentiated startle paradigm is also mediated by amygdaloid glutamate receptors (Kim et al., 1993), it is conceivable that the blockade of retrieval of the fear memory by 30 mg/kg MPEP also involved the amygdala. The assumption that MPEP blocked fear-learning in the amygdala is supported by recent data showing that intraamygdalar infusion of MPEP impairs the acquisition but not the expression of conditioned fear (Fendt et al., 2000). It has to be noted, though, that the expression of fear-potentiated startle is also dependent on glutamate receptors in the caudal pontine reticular nucleus, an essential part of the primary ASR circuit (Fendt et al., 1996), but our local infusion data suggest that MPEP did not act at the level of the brainstem.

4.2. Role of mGluR5 in sensorimotor gating

PPI is the reduction in ASR magnitude that occurs when a weak prepulse is presented shortly before the startling noise pulse is given (Hoffman and Ison, 1980). PPI is an operational measure of sensorimotor gating mechanisms of the brain whose function is to suppress inadvertent responding. Reductions in PPI are observed in some neuropsychiatric disorders (e.g. schizophrenia and Huntington's disease) and, therefore, experimentally induced PPI-deficits in rats have been considered to model some aspects of these disorders (Swerdlow and Geyer, 1998). PPI can be reliably reduced by the systemic or intracerebral administration of competitive and non-competitive NMDA receptor antagonists (Koch, 1999). Our present findings clearly show that a blockade of mGluR5 in the brain does not impair sensorimotor gating as measured by PPI. This finding is supported by a recent paper showing that the group I antagonist (+) MCPG did not affect PPI (Grauer and Marquis, 1999). Interestingly, in this latter paper it was also reported that PPI was reduced by intrastriatal infusions of group I and II mGluR agonists and that the PPI-deficit induced by intraaccumbal trans-ACPD was antagonised by (+) MCPG. Since this suggests a potential antipsychotic potency of mGluR antagonists, future studies will investigate the effects of MPEP on drug-induced PPI-deficits. The finding that the pharmacological blockade of mGluR5 by MPEP has no effect on PPI is in slight contrast with the observation that mGluR5 knockout mice show a profound PPI-deficit (Henry et al., 1999). It is presently unclear if this is due to a species difference or based on the fact that a constitutive knockout eliminates the receptor in all somatic cells throughout the whole ontogeny, whilst the pharmacological blockade of the receptor acutely impairs its function in an intact adult animal.

Taken together, the present data show that the selective mGluR5 antagonist MPEP impairs the acquisition and the expression of conditioned fear. The latter effect can be interpreted as an anxiolytic effect of the compound. No effect of MPEP on the immediate perception of footshocks was found, suggesting that the impairment of fear conditioning reflects a specific learning deficit by blockade of mGluR5. Finally, PPI as a measure of sensorimotor gating was not impaired by MPEP.

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Blockade of the metabotropic glutamate receptor subtype 5 (mGluR5) produces antiparkinsonian-like effects in rats

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Abstract

The aim of the present study was to examine a potential beneficial effect of the blockade of metabotropic glutamate receptor subtype 5 (mGluR5) by the selective non-competitive antagonist, 2-methyl-6-(phenylethynyl)pyridine (MPEP), in models of parkinsonian symptoms in rats. Haloperidol, 0.25, 0.5 and 1 mg/kg ip, was used to induce hypolocomotion, catalepsy and muscle rigidity, respectively. The locomotor activity was estimated by an open-field test, the catalepsy — by a 9-cm cork test. The muscle rigidity was measured as an increased resistance of a hind leg to passive extension and flexion at the ankle joint. Additionally, increases in the electromyographic activity were recorded in the gastrocnemius and tibialis anterior muscles. MPEP (1.0–10 mg/kg ip) inhibited the muscle rigidity, electromyographic activity, hypolocomotion and catalepsy induced by haloperidol. MPEP administered alone (5 mg/kg ip) did not induce catalepsy, nor did it influence the muscle tone or locomotor activity in rats. The present results suggest that blockade of mGluR5 receptors may be important to amelioration of both parkinsonian akinesia and muscle rigidity.

Keywords: mGluR5 glutamate receptors; 2-Methyl-6-(phenylethynyl)pyridine; Antiparkinsonian-like effects; Locomotor activity; Catalepsy; Muscle rigidity

1. Introduction

The primary cause of Parkinson's disease is degeneration of dopaminergic neurons in the substantia nigra pars compacta, which results in a dramatic decrease in dopamine content in the corpus striatum. It has been suggested that this effect triggers off a number of secondary neuronal alterations which finally lead to the glutamate-induced overexcitation of neurons localized in the latter structure, as well as in the subthalamic nucleus, substantia nigra pars reticulata, and globus pallidus pars interna (Klockgether and Turski, 1989; Ossowska, 1994; Biandini et al., 2000). The role of glutamatergic overactivity in the appearence of parkinsonian symptoms has been supported by a number of studies which suggest that antagonists of *N*-methyl-D-aspartate (NMDA) receptors induce antiparkinsonian effects in both rodent models and Parkinson's disease (Klockgether and Turski, 1990; Ossowska, 1994; Yoshida et al., 1994; Kretschmer and Schmidt, 1996; Ossowska and Konieczny, 1996; Kaur and Starr, 1997; Lorenc-Koci et al., 1998).

Glutamate acts not only on ionotropic, but also on metabotropic receptors (mGluRs) which have been divided into three groups. Group I contains 2 receptors (mGluR1 and 5). Their stimulation activates phospolipase C and phosphoinositide hydrolysis, and increases neuronal excitability. In contrast, stimulation of group II (mGluR2 and 3) and III receptors (mGluR4, 6, 7 and 8) decreases adenylate cyclase activity and cAMP level, as well as glutamate release via autoreceptors (Schoepp et al., 1999; Pilc et al., 1998).

Our recent studies (Konieczny et al., 1998) showed for the first time that drugs acting on mGluRs may be important to the treatment of parkinsonian symptoms. We found that the selective agonist of group II mGluRs, (+)-2-aminobicyclo[3.1.0.]hexane-2,6,-dicarboxylic acid (LY 354,740), administered systemically inhibited the parkinsonian-like muscle rigidity induced by haloperidol in rats. In subsequent studies, Bradley et al. (2000) and

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Dawson et al. (2000) reported antiakinetic effects of LY 354,740 and (2*S*,2′*R*,3′*R*)-2-(2′,3′dicarboxycyclopropyl) glycine (DCG-IV; another agonist of those receptors) in two rat models: the haloperidol-induced catalepsy and the reserpine-induced akinesia.

Recent studies have also provided some arguments for potential significance of mGluR5 blockade for antiparkinsonian effects. Awad et al. (2000) found that stimulation of group I mGluRs produced excitation of neurons in the subthalamic nucleus, which was inhibited by the mGluR5 non-competitive antagonist, 2-methyl-6-(phenylethynyl)pyridine (MPEP). Moreover, Spooren et al. (2000) showed that MPEP induced ipsilateral rotations in rats whose nigrostriatal dopamine pathway was unilaterally lesioned with 6-OHDA.

In the present study we examined potential beneficial effects of MPEP in rat models of some parkinsonian symptoms: the haloperidol-induced akinesia and muscle rigidity.

2. Methods

2.1. Animals

The experiments were carried out in compliance with the Animal Protection Bill of August 21, 1997; (published in Dziennik Ustaw no. 111/1997 item 724), and according to the NIH guide for the Care and Use of Laboratory Animals.

Male Wistar rats weighing 190–250 g (for catalepsy or locomotor activity studies) or 300–370 g (the mechano-electromyographic measurement) were kept on an artificial light/dark cycle (12/12 h; the light on from 7 a.m. to 7 p.m.) with free access to food and water.

2.2. Mechano- and electromyographic measurement

Simultaneous measurement of muscle resistance of a rat's hind leg (MMG) and an electromyographic activity of muscles (EMG), developed in response to passive movements, were recorded as described previously (Ossowska et al., 1996a; Lorenc-Koci et al., 1996). Each rat was placed in a metaplex cage, well-ventilated and adapted to its size. A rat's hind foot was pulled out of the cage through an opening at the bottom of the cage, placed on the appropriately matched metaplex block, and gently fixed to it with an adhesive tape. Two pairs of flexible stainless steel wire electrodes (Cooner Wire, Chatsworth, CA, USA), which were Teflon-insulated (external diameter — 0.25 mm) except for a 4-mm uninsulated part (external diameter 0.1 mm), were inserted percutaneously into the gastrocnemius (extensor, plantar flexor) and tibialis anterior (flexor, dorsal flexor) muscles. The distance between the two electrodes of a pair in each muscle was ca. 5 mm. An earth electrode was gently attached with a clip to a rat's tail covered with a special electrode cream.

The experiment involved successive movements of the block (30 s apart) which passively extended and flexed a rat's leg at the ankle joint by 25°. Each movement lasted 250 ms. The metaplex block was connected to a force sensor which recorded the resistance of the leg to passive movements (a mechanical moment, torque, MMG). EMG signals from the electrodes were amplified and band-pass-filtered (80 Hz to 10 kHz; Polygraph Grass, model 78). The recording of EMG and MMG signals started 200 ms earlier, and was carried out for 250 ms throughout and 550 ms after the termination of each passive movement. EMG and MMG signals were sampled with analog-digital converters (ADs) at a frequency of 10 kHz per channel, and were fed into a PC.

The maximum resistance of a rat's hind leg muscles, developed in response to each movement, was estimated. The EMG activity of the gastrocnemius and tibialis anterior muscles was rectified and integrated with a time constant of 20 ms for each movement. All the data disturbed by voluntary movement artefacts were discarded. In order to visualize the mean tendency in each group of animals, all the undisturbed, rectified and integrated EMG curves for either muscle (gastrocnemius or tibialis) and movement (flexion or extension) were superimposed.

2.3. Catalepsy

Catalepsy was determined using a 9-cm cork test. Both forepaws of a rat were put on the cork. Rats were regarded as cataleptic when they kept at least one paw on the cork for more than 30 s.

2.4. Open field test

The 'open field' apparatus was a circle made of wood, 90 cm in diameter, divided into six triangles with pale white lines. Each animal was placed singly in the centre of the 'open field'. The time of walking (in s) was measured during a 5-min observation period. The experiments were performed in a dark room and the apparatus was illuminated with a 60 W bulb positioned 1 m above the centre of the circle.

2.5. Drugs and experimental schedule

Haloperidol (RBI, Natick, MA, USA) was dissolved in a small volume of a 1% lactic acid, diluted to a final concentration with distilled water. 2-methyl-6-(phenylethynyl)pyridine (MPEP, Novartis Pharma AG, Basel, Switzerland) was dissolved in distilled water. Control groups received physiological saline instead of haloperidol and/or MPEP.

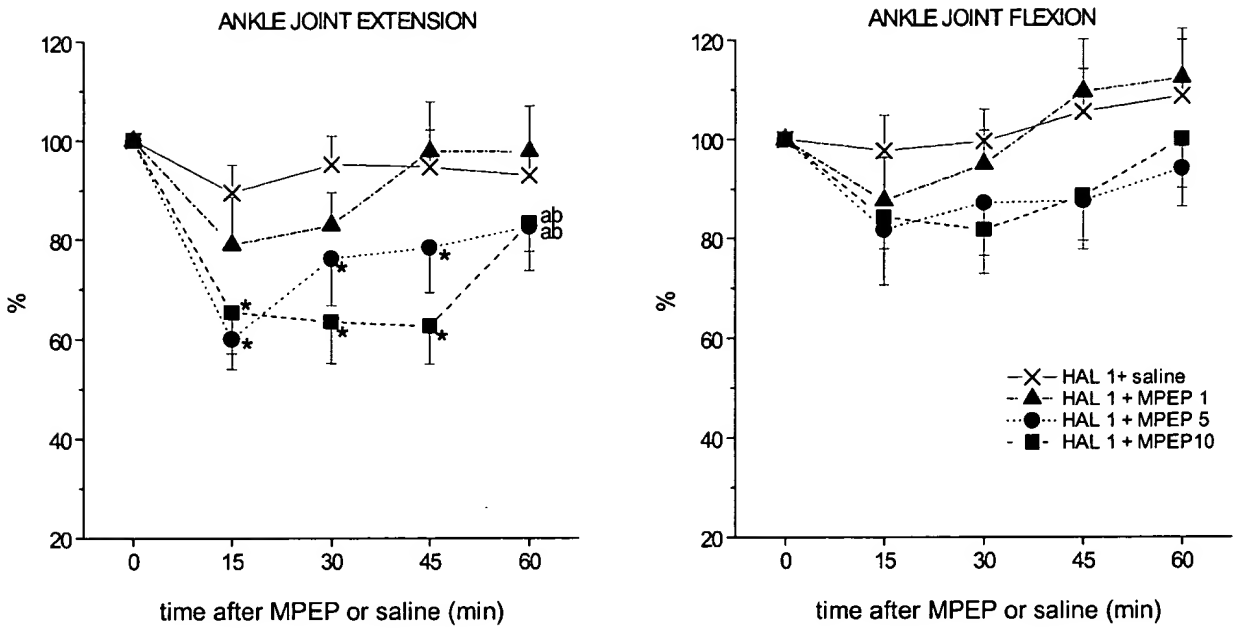

Fig. 1. The influence of MPEP (1–10 mg/kg ip) on the muscle rigidity induced by haloperidol (HAL, 1 mg/kg ip) in rats. MPEP was administered 60 min after HAL. Muscle tone was measured as a maximum resistance of a hind leg to passive extension and flexion at the ankle joint (max. torque). The mean value of the muscle resistance to flexion and extension, measured 30–60 min after HAL administration, was accepted as 100% for each rat. The data are shown as a percentage of this value (mean±SEM). Abscissa — time after MPEP/or saline administration (in min); The number of animals in groups: HAL 1+saline — n=8, HAL 1+MPEP 1 — n=7, HAL 1+MPEP 2.5 — n=7, HAL 1+MPEP 5 — n=9, HAL 1+MPEP 10 — n=8. Statistics — a two-way ANOVA and the Newman–Keuls post hoc test; significant differences throughout the whole registration period: a — vs HAL 1+saline (p<0.01–0.05); b — vs HAL 1+MPEP 1 (p<0.03–0.05). Significant differences at individual time points: * — vs HAL 1+saline. For clarity of the picture, a dose of 2.5 mg/kg of MPEP was discarded.

2.5.1. Mechano- and electromyographic experiments

The rats were treated with haloperidol (1 mg/kg ip), and after 15 min they were put into a metaplex cage for a 15-min adaptation period, during which passive movements of the hind foot were executed, but no recording was carried out. Mechano- and electromyographic measurements started 30 min after haloperidol. MPEP (1, 2.5, 5 or 10 mg/kg ip) or saline were injected 60 min after haloperidol (30 min after the start of measurements). Recordings were continued for 60 min after MPEP administration (60–120 min after haloperidol). The number of the animals tested was 5–9/group.

2.5.2. Catalepsy

The rats were pretreated with haloperidol (0.5 mg/kg ip). MPEP (2.5, 5 or 10 mg/kg ip) was injected 60 min after haloperidol. Catalepsy was estimated twice: 30 and 60 min after MPEP. The number of the animals tested was 5–8/group.

2.5.3. Open field test

Haloperidol was administered in a dose of 0.25 mg/kg ip, and MPEP in a dose of 5 mg/kg ip. Two different schedules were used: (1) MPEP was administered 30 min after haloperidol and the test was performed 30 min thereafter (60 min after haloperidol); (2) MPEP was injected 5 min before haloperidol and the test was carried out 30 min after haloperidol (35 min after MPEP). The number of the animals tested was 7–16/group.

2.6. Statistics

An analysis of muscle resistance was carried out using the means obtained from all the correct passive movements calculated: (1) during a 30-min period between the end of adaptation and MPEP injections (30–60 min after haloperidol) and (2) every 15 min after MPEP or saline administration. The mean obtained before MPEP administration was accepted as 100% for each rat, and all the successive data were shown as a percentage of that value. A two-way ANOVA, followed — when significant — by the Newman–Keuls post hoc test, was used for a further data analysis. A two-way ANOVA and the Newman–Keuls tests were also used for the estimation of open-field data.Catalepsy data were shown as a percentage of cataleptic rats, and the significance of differences was checked using a χ^2 test.

3. Results

3.1. Mechanographic and electromyographic measurements

As had already been described earlier (Lorenc-Koci et al., 1996), haloperidol (1 mg/kg) induced muscle

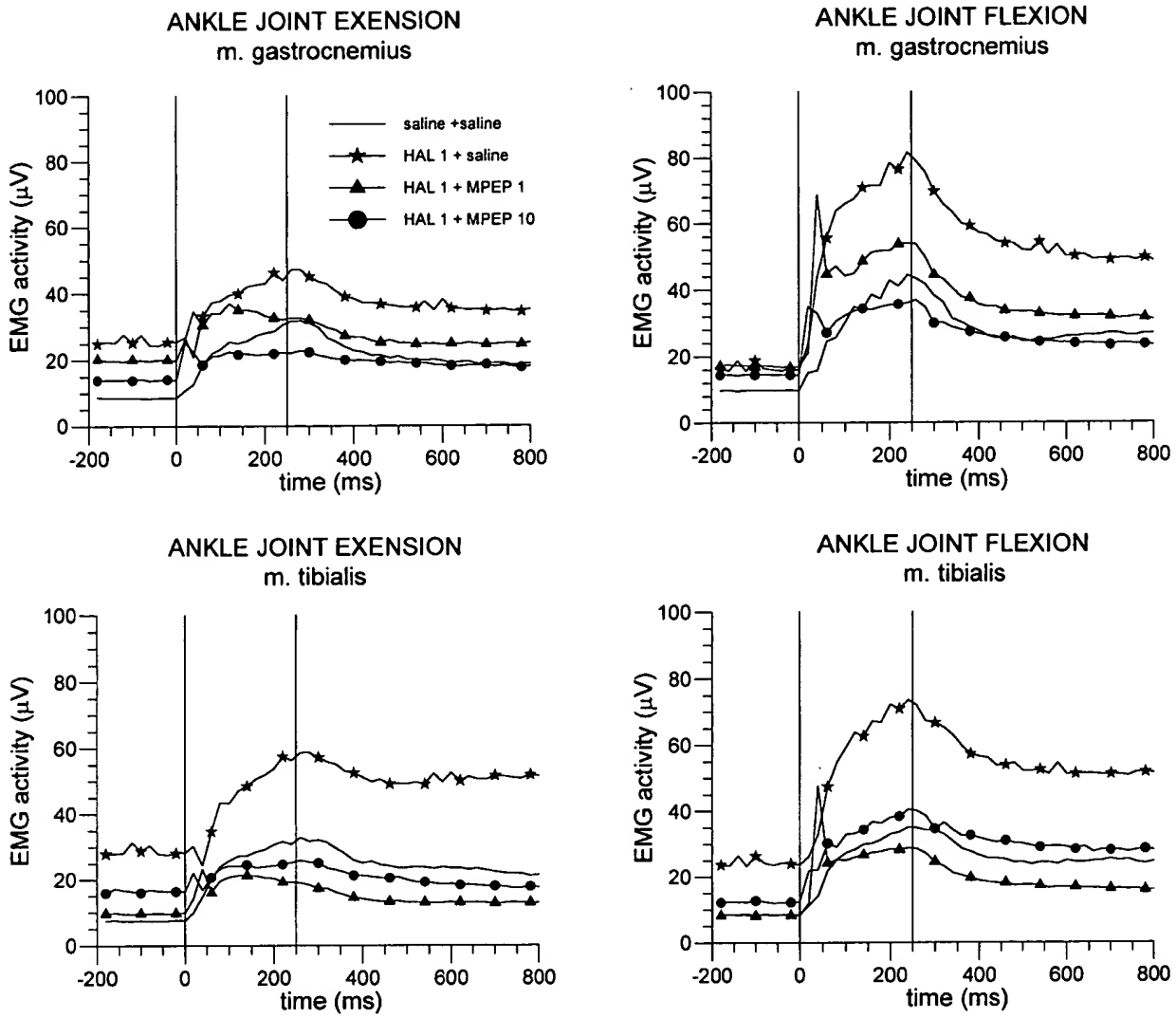

Fig. 2. The influence of MPEP (1–10 mg/kg ip) on the electromyographic (EMG) activity increased by haloperidol (HAL, 1 mg/kg ip) in rats. HAL was administered 60 min before MPEP. The EMG activity was recorded in the gastrocnemius and tibialis anterior muscles during passive extension and flexion of a hind foot at the ankle joint. Curves represent the EMG activity which was rectified, integrated and superimposed for a 60-min registration period. Ordinate — EMG activity in μV; abscissa — time of a movement in ms (the start and the end of a movement is indicated with vertical lines). For the clarity of the picture, only two doses (1 and 10 mg/kg) of MPEP are shown. The number of animals in groups see Fig. 1.

rigidity, measured as an increased muscle resistance of rats' hind legs, developed in response to passive extension [treatment effect: $F(1,56)=120$, $p<0.001$; non-significant time effect; non-significant treatment×time interaction] and flexion [treatment effect: $F(1,56)=156$, $p<0.001$; non-significant time effect; non-significant treatment×time interaction] at the ankle joint (data not shown). MPEP (1–10 mg/kg), administered 60 min after haloperidol, decreased in a dose-dependent manner the muscle rigidity induced by that neuroleptic (Fig. 1). A two-way ANOVA, used for evaluation of the extension data, showed a significant treatment effect [$F(4,132)=7.2$, $p<0.001$], a non-significant time effect, and no treatment×time interaction. A two-way ANOVA, used to assess the flexion data, showed a treatment effect close to the borderline of statistical significance [$F(4,132)=2.38$, $p=0.0551$], a significant time effect [$F(3,132)=2.79$, $p<0.05$] and no treatment×time interaction. Treatment with MPEP (5 mg/kg; $n=5$) alone did not significantly influence the muscle tone of rats measured during flexion or extension [non-significant: treatment effect, time effect, and treatment×time interaction] (data not shown).

Inspection of the rectified and averaged EMG curves confirmed our earlier data (Lorenc-Koci et al., 1996), and showed that haloperidol (1 mg/kg) increased the EMG activity of the gastrocnemius and tibialis anterior muscles measured before, during and after each move-

ment (flexion and extension) (Fig. 2). MPEP (1–10 mg/kg), administered 60 min after haloperidol, decreased the haloperidol-increased EMG activity. However, no clear-cut dose-dependent effect was observed (Fig. 2). MPEP (5 mg/kg; n=5) administered alone did not influence the EMG activity in naïve rats (data not shown)

3.2. Catalepsy

MPEP (5 and 10 mg/kg), administered 60 min after haloperidol (0.5 mg/kg), significantly decreased the catalepsy induced by that neuroleptic (Fig. 3). Although no difference in the potency of those doses was observed at 30 min after MPEP administration, the effect of the higher dose lasted longer and was still significant 30 min later. The lowest dose tested of MPEP (2.5 mg/kg) did not significantly influence the haloperidol-induced catalepsy. However, a slight, insignificant trend towards inhibition was observed 30 min after MPEP administration. MPEP (5 mg/kg) administered alone did not induce catalepsy (Fig. 3).

3.3. Open field test

Haloperidol (0.25 mg/kg) induced hypolocomotion which was evidenced by a decrease in the time of walking, measured 30 (Fig. 4B) and 60 min (Fig. 4A) after its administration. A two-way ANOVA showed a significant effect of this treatment [$F(1,58)=50.2$; $p<0.001$ (30 min) (Fig. 4B); $F(1,25)=45.95$; $p<0.001$ (60 min) (Fig. 4A)]. The two-way ANOVA showed additionally a significant effect of MPEP (5 mg/kg) [$F(1,25)=16.96$; $p<0.001$ (Fig. 4A); $F(1,58)=8.3$; $p<0.01$ (Fig. 4B)]. However, the latter was not due to an influence of MPEP on the locomotor activity of naive rats, since a post hoc comparison (the Newman–Keuls test) did not show any significant difference between MPEP-treated and control, saline-treated animals (Fig. 4A and B).

In contrast, MPEP antagonized the hypolocomotion induced by haloperidol when it was given either 30 min after [haloperidol×MPEP interaction: $F(1,25)=6.3$; $p<0.01$ (Fig. 4A)] or 5 min before that neuroleptic [haloperidol×MPEP: $F(1,58)=5.4$; $p<0.02$ (Fig. 4B)]. The latter finding was supported by significant differences between groups of rats treated with haloperidol alone and those treated jointly with haloperidol and MPEP ($p<0.001$; the Newman–Keuls test) (Fig. 4A and B).

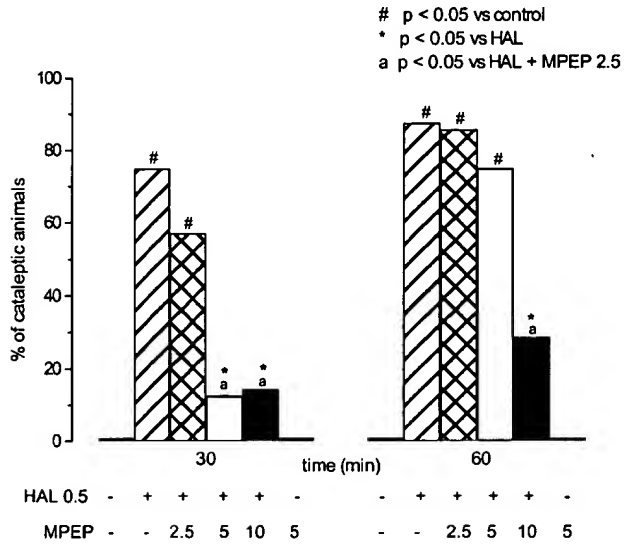

Fig. 3. The influence of MPEP (2.5–10 mg/skg ip) on the catalepsy induced by haloperidol (HAL, 0.5 mg/kg ip). No cataleptic effect was shown for MPEP alone (5 mg/kg ip). The results are shown as a percentage of cataleptic rats. MPEP was administered 60 min after HAL. The number of animals in groups: control (saline+saline) — n=5, HAL 0.5+saline — n=8, HAL 0.5+MPEP 2.5 — n=7, HAL 0.5+MPEP 5 — n=8, HAL 0.5+MPEP 10 — n=7, saline+MPEP 5 — n=5. Statistics — χ^2 test.

4. Discussion

The present study shows that the blockade of mGluR5s by the selective antagonist MPEP produces antiparkinsonian-like effects in rats. Such a conclusion can be drawn from the findings showing that MPEP antagonizes the haloperidol-induced catalepsy, hypolocomotion and muscle rigidity.

The catalepsy induced by neuroleptics consists in the inability of an animal to change an uncomfortable position imposed by the experimenter. The catalepsy, as well as the neuroleptic-evoked hypolocomotion are widely accepted as models of akinesia observed in parkinsonian patients who are slow and unable to change their motor programme. Our recent studies suggest that haloperidol may also model the parkinsonian muscle rigidity (Lorenc-Koci et al., 1996). The muscle rigidity present in the course of Parkinson's disease is characterized by an increase in the muscle resistance of a patient's extremities, estimated during their passive displacement, as well as by enhancement of the resting and reflex EMG activities in the examined muscles (Lee, 1989). We observed that haloperidol increased both the muscle resistance of a rat's hind leg and EMG activity recorded before (resting activity) and during its passive movements (reflex related activity) (Lorenc-Koci et al., 1996; present results). Moreover, all the above-mentioned haloperidol-induced effects were inhibited by some antiparkinsonian agents (L-DOPA, pramipexol) (Lorenc-Koci and Wolfarth, 1999; Wardas et al., 2001).

Recently, Spooren et al. (2000) have reported that MPEP produces ipsilateral rotations in unilaterally 6-hydroxydopamine-lesioned rats, which may speak for an antiparkinsonian-like action of this compound. However, the latter effect is rather weak. Moreover, MPEP inhibits

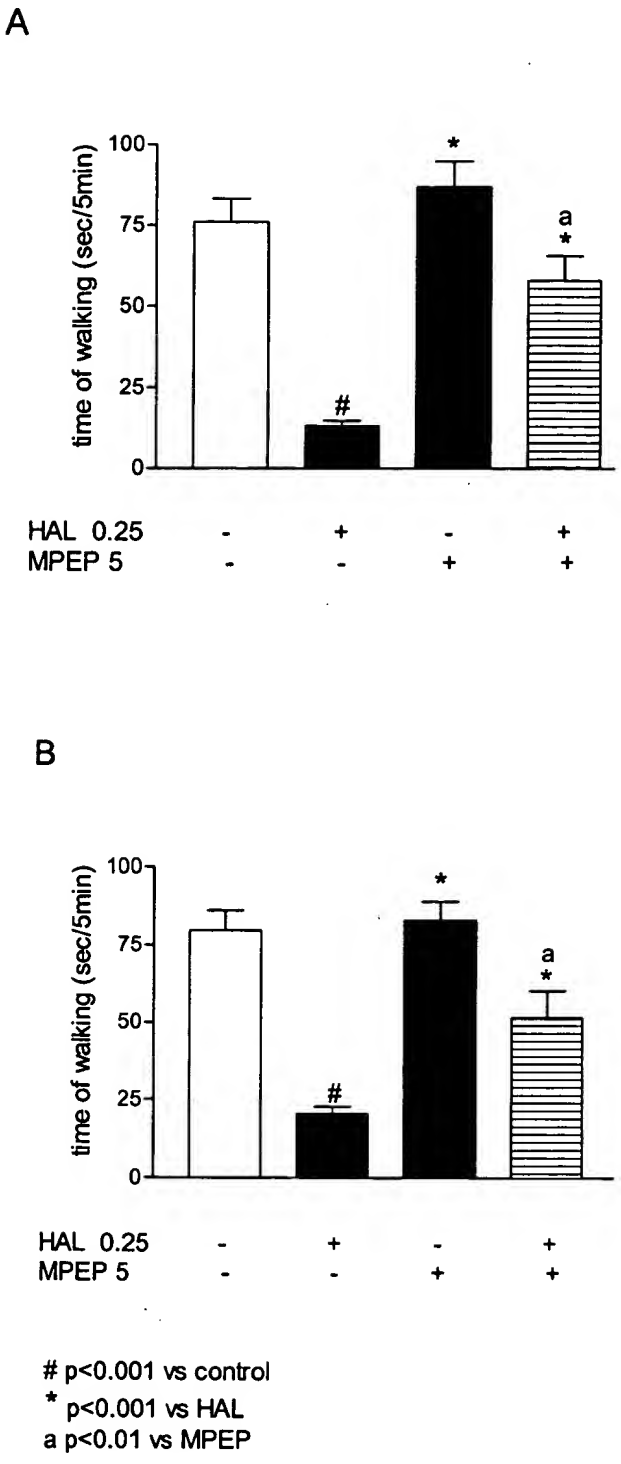

Fig. 4. The influence of MPEP (5 mg/kg ip) on the hypolocomotion induced by haloperidol (HAL, 0.25 mg/kg ip). Locomotor activity was estimated in the open field test as the time of walking (in s) during a 5-min observation period. (A) MPEP was administered 30 min after HAL. The test was carried out 30 min after MPEP (60 min after HAL); The number of animals in groups: control (saline+saline) — n=7, HAL+saline — n=7, HAL+MPEP — n=7, saline+MPEP — n=8. (B) MPEP was administered 5 min before HAL. The test was carried out 35 min after MPEP (30 min after HAL). The number of animals in groups: control (saline+saline) — n=16, saline+HAL — n=15, MPEP+HAL — n=15, MPEP+saline — n=16. Statistics — a two-way ANOVA and the Newman–Keuls post hoc test.

the rotational behavior induced by dopaminomimetics (Spooren et al., 2000). Therefore the above-quoted authors have concluded that MPEP, either given alone or in combination with dopaminomimetics, may not have a great impact on the treatment of parkinsonian symptoms in humans. However, since the present study shows that MPEP inhibits muscle rigidity, EMG hyperreflexibility, hypolocomotion and catalepsy, i.e. symptoms directly related to parkinsonism, the reported conclusion of Spooren et al. (2000) seems to be premature.

Since in the present study MPEP was administered systemically, the target of its antiparkinsonian-like effects is not clear. At least three brain structures that receive glutamatergic innervation, i.e. the striatum, subthalamic nucleus, substantia nigra pars reticulata and internal part of the globus pallidus may be involved (Klockgether and Turski, 1990; Ossowska, 1994). MGluR5s are present in all these structures (Testa et al. 1994, 1995; Ghazemzadeh et al., 1996; Kerner et al., 1997; Thallaksen-Greene et al., 1998), their high expression being found in the striatum and moderate in other brain structures (Testa et al., 1994). The role of glutamate receptors localized in all the above-mentioned structures in the action of antiparkinsonian drugs had been suggested previously, since direct injections of NMDA receptor antagonists into these regions produced beneficial effects in rat models of parkinsonism (Klockgether and Turski, 1990; Ossowska, 1994; Yoshida et al., 1994; Kretschmer and Schmidt, 1996; Ossowska and Konieczny, 1996; Kaur and Starr, 1997; Lorenc-Koci et al., 1998). Our recent studies also showed that direct intrastriatal administration of the selective antagonist of mGluR1, (*R,S*)-1-aminoindan-1,5-dicarboxylic acid (AIDA), or the mixed group I antagonist/group II agonist, (*S*)-4-carboxy-3-hydroxyphenyl-glycine ((*S*)-4C3HPG), inhibited the haloperidol-induced muscle rigidity (Wolfarth et al., 2000; Lorenc-Koci et al., 2001). MGluRs that belong to group I are co-localized with NMDA receptors on the same striatal neurons, and they modulate positively the NMDA-increased neuronal excitability in the striatum (Ghazemzadeh et al., 1996; Pisani et al., 1997). Therefore it may be supposed that their blockade by, for example, MPEP, produces behavioural effects similar to the blockade of striatal NMDA receptors. However, an influence of MPEP on other extrastriatal mGluR5s is also possible, since this compound has been found to inhibit the neuronal activity of the subthalamic nucleus, increased by stimulation of group I mGluRs (Awad et al., 2000). The overactivity of glutamatergic pathways which lead from the subthalamic

nucleus to the substantia nigra pars reticulata and the internal part of the globus pallidus is crucial for the appearance of parkinsonian symptoms (Klockgether and Turski, 1989; Bergman et al., 1990; Ossowska, 1994; Biandini et al., 2000); hence the above-described effect of MPEP might be important for its potential antiparkinsonian action.

MPEP has been suggested to be a selective, non-competitive mGluR5 antagonist which blocks these receptors at nanomolar concentrations (Schoepp et al., 1999). However, recent studies indicate that MPEP at micromolar concentrations also antagonizes the NMDA-induced responses (O'Leary et al., 2000; Movsesyan et al., 2001). Nevertheless, significant contribution of the blockade of NMDA receptors to the above-mentioned antiparkinsonian-like effects of this compound seems unlikely. Antagonists of NMDA receptors (e.g. MK-801), administered to rats in doses which inhibit parkinsonian-like symptoms (catalepsy, hypolocomotion, increased muscle resistance, or EMG activities) (Schmidt and Bubser, 1989; Klockgether and Turski, 1990; Schmidt et al., 1992; Ossowska et al., 1994, 1996b), produce locomotor stimulation, ataxia and myorelaxation, which suggests a possibility of appearance of serious undesirable side-effects in humans (Hiramatsu et al., 1989; Löscher and Hönack, 1991; Ossowska, 1994; Andiné et al., 1999). In contrast, both recent studies (Spooren et al., 2000) and the present one have shown that MPEP, administered in doses which produce antiparkinsonian-like effects, does not induce ataxia or myorelaxation, nor does it increase locomotor activity in rats. The lack of these effects promises well for the future regarding human therapy with this agent.

Our previous study (Konieczny et al., 1998), as well as that of Bradley et al. (2000) showed that the selective agonist of group II mGluRs, LY 354,740, also inhibited the haloperidol-induced muscle rigidity and catalepsy. The latter effects may have resulted from activation of autoreceptors, since LY 354,740 was found to block the veratridine-evoked glutamate release in the striatum (Battaglia et al., 1997), and to reduce the excitation of nigral neurons induced by stimulation of the subthalamonigral pathway (Bradley et al., 2000).

Summing up, the above-cited data suggest that antiparkinsonian effects may be achieved by inhibition of the glutamatergic transmission at two levels: (1) at a level of postsynaptic group I mGluR, or NMDA receptors whose blockade decreases neuronal excitation and (2) at a level of group II mGluR autoreceptors whose activation decreases glutamate release. These findings open up new perspectives in the search for new, potential antiparkinsonian drugs among the above-described classes of compounds.

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Effects of the prototypical $mGlu_5$ receptor antagonist 2-methyl-6-(phenylethynyl)-pyridine on rotarod, locomotor activity and rotational responses in unilateral 6-OHDA-lesioned rats

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Abstract

In the present study, we evaluated the effect of the prototypical metabotropic glutamate receptor 5 ($mGlu_5$) antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP) on motor behaviour in rats using the accelerating rotarod, spontaneous locomotor activity and the 6-hydroxy-dopamine (6-OHDA) lesion model to assess its treatment potential for Parkinson's disease. The data indicate that MPEP at doses between 7.5 and 300 mg/kg, p.o. did not disrupt endurance performance on the accelerating rotarod (4–40 rpm in 300 s) which indicates that MPEP has a relatively high safety margin. However, while ineffective at doses of 3.75, 7.5 and 15 mg/kg (p.o.) MPEP inhibited spontaneous locomotor activity at doses of 30 and 100 mg/kg (p.o.). In the 6-OHDA rat rotation model, at doses of 7.5, 15 and 30 mg/kg (p.o.), MPEP induced a dose-dependent ipsilateral rotational response that reached statistical significance at the highest dose tested. This effect was relatively small but consistent. In combination with direct or indirect dopamine agonists, i.e. apomorphine (0.25 mg/kg, s.c.) and D-amphetamine (2.5 mg/kg, i.p.), MPEP (7.5, 15 or 30 mg/kg, p.o.) was found to significantly inhibit these dopamine receptor mediated rotational responses. MPEP injected at a dose of 30 mg/kg also inhibited the rotational response induced by L-DOPA (25 mg/kg, i.p.). (+)MK-801 was used in these rotation experiments as the reference compound. In view of these findings, it could be concluded that MPEP and potentially other $mGlu_5$ receptor antagonists are probably not appropriate drug candidates for the symptomatic treatment of Parkinson's disease.

Keywords: Glutamate; Receptor; Metabotropic; Antagonist; 2-Methyl-6-(phenylethynyl)-pyridine (MPEP)

1. Introduction

Metabotropic glutamate receptors are a family of G-protein-coupled receptors linked to multiple second messengers and modulation of ion channel functions in the nervous system (Knöpfel et al., 1995; Conn and Pinn, 1997). Molecular cloning has revealed the existence of eight distinct receptor subtypes, termed $mGlu_1$–$mGlu_8$, which are classified into three subgroups based on sequence similarities, pharmacological profiles and signal transduction pathways activated in heterologous systems. Group I receptors ($mGlu_1$ and $_5$) couple to phospholipase C and regulate neuronal excitability whereas group II ($mGlu_2$ and $_3$) and group III receptors ($mGlu_4$, $_6$, $_7$, $_8$) inhibit adenylyl cyclase and modulate neurotransmitter release.

Metabotropic glutamate receptors have been proposed as potentially new therapeutic targets for a number of neurological and psychiatric disorders (Knöpfel et al., 1995; Conn and Pinn, 1997; Nicoletti et al., 1997). However, these speculations are largely based on the expression pattern of distinct mGlu-subtype receptors in the central nervous system and on the effects of non-selective compounds that fail to discriminate between distinct receptor subtypes. The recent discovery of a series of potent, selective and systemically active antagonist for the $mGlu_5$ receptor opened now the possibility to investigate the role of this receptor subtype in behaviour and disease states (Gasparini et al., 1999; Varney et al., 1999). The most potent derivative of this series, 2-methyl-6-(phenylethynyl)-pyridine (MPEP), completely inhibited quisqualate-stimulated phosphoinositide hydrolysis with an IC_{50} value

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of 36 nM whilst devoid of agonist or antagonist activities at any of the other mGlu receptor subtypes up to 100 μM (Gasparini et al., 1999). Upon testing in rodents it was found that MPEP is readily oral bioavailable and easily penetrates the brain reaching relatively high brain levels shortly (within 60 min) following oral application (Spooren et al., unpublished observation).

The $mGlu_5$ receptor is widely distributed in the central nervous system with particularly high expression in hippocampus and the striatal areas such as the nucleus accumbens and caudate putamen. However, $mGlu_5$ receptors are also expressed in the output structures of the striatum, i.e. the internal and external pallidal segments and the substantia nigra pars reticulata (Testa et al., 1994). These brain areas are well-known as key elements in the basal ganglia circuitry and, therefore, to play an important role in behaviour and disease such as in Parkinson's disease (Albin et al., 1989).

A widely used model to evaluate the potential antiparkinson activity of experimental therapeutics is the so-called hemiparkinsonian rat or the unilateral 6-OHDA lesioned rat (Ungerstedt, 1971; for review: Schwarting and Huston, 1996a,b). The model involves the unilateral injection of 6-OHDA into the medial forebrain bundle which induces extensive loss of dopaminergic cells in the substantia nigra pars compacta (Schwarting and Huston, 1996a,b). The resulting imbalance in dopamine innervation between the striata produces postural asymmetry. Dopamine releasing agents such as amphetamine exacerbate the dopamine imbalance that favors the non-lesioned side and thus produces ipsilateral rotations (Schwarting and Huston, 1996a,b). In contrast, direct agonists such as apomorphine evoke contralateral rotations reflecting an action at supersensitive denervated dopamine receptors ipsilateral to the lesion (Schwarting and Huston, 1996a,b). In the present study, we have used the 6-OHDA model to investigate the therapeutic potential of the novel prototypical $mGlu_5$ receptor antagonist MPEP for the symptomatic treatment of Parkinson disease. We here describe the effect of MPEP alone as well as when given in a combination with apomorphine, D-amphetamine and L-DOPA. In addition, the effect of MPEP on motor coordination and motor activity was further investigated using the accelerating rotarod and spontaneous locomotor activity, respectively.

2. Materials and methods

2.1. Rotarod and locomotor activity experiments

2.1.1. Animals

Male Wistar rats (Iffa Credo, Les Oncins, France; $n = 222$), 100–120 g were used. The animals were housed four per cage in a temperature controlled room (22 ± 1°C) under artificial illumination (6:00–18:00 h, lights on) with access to water and food (Ecosan, Eberle Nafag, Gossau, Switzerland), ad libitum.

2.1.2. Rotarod

Drug-naïve animals were trained twice daily on two successive days on the accelerating rotarod (4–40 rpm in 300 s; TSE, Bad Homburg, Germany). On the test day (day 3), the animals received an injection of MPEP at low doses (7.5, 15, or 30 mg/kg, p.o.; $n = 12$ per treatment group) or vehicle (methylcellulose (0.5%); Animed, Allschwil, Switzerland) or an injection of MPEP at high doses (100, 200 or 300 mg/kg, p.o.), baclofen (10 mg/kg, p.o., i.e. a dose known to reduce endurance performance; Spooren et al. unpublished observation) as the positive control, or vehicle. The animals were then repeatedly tested for their endurance performance on the accelerating rotarod (longest time spent on the rotarod; cut of time 300 s) 1, 3, 6 and 24 h after application.

2.1.3. Locomotor activity

Drug-naïve non-habituated rats received an injection with MPEP at doses 3.75, 7.5, 15, 30 or 100 mg/kg, p.o., or the vehicle (methylcellulose (0.5%); $n = 18$ per treatment group) and they were subsequently placed in locomotor activity cages (17 × 32 × 20 cm; Motron motility, Novartis Pharma, Basel, Switzerland) and the number of beam interruptions (vertical and horizontal) was registered in 10-min intervals for 120 min.

2.2. 6-OHDA rat rotation experiments

2.2.1. Animals

Male Sprague–Dawley Rats (Iffa Credo, Les Oncins, France; $n = 120$), 250–280 g at the time of surgery (see below) were used. The animals were housed four per cage in a temperature controlled room (22 ± 1°C) under artificial illumination (6:00–18:00 h, lights on) with access to water and food (Ecosan, Eberle Nafag), ad libitum.

2.2.2. Surgery

Procedures were outlined previously (Spooren et al., 1999) and adapted from Nitsch et al. (1993). Briefly, before surgery all animals received an injection of desipramine hydrochloride (30 mg/kg, i.p.; USPC, Rockville, USA) to protect noradrenergic cells. One hour later the animals received an injection of pentobarbital (55 mg/kg, i.p., Vetanarcol, Veterinaria, Zurich, Switzerland) and the animals were subsequently placed (under deep anesthesia) in a UHL stereotaxic apparatus. A unilateral lesion was made by injecting 9 μg 6-OHDA (6-OHDA-hydrobromide; Fluka Chemie, Buchs, Switzerland) in 0.7 μl ascorbic acid solution (dilution: 1 mg/ml) over 10 min into the left medial forebrain bundle (coordinates: AP 3.6 mm (from bregma), L 1.1 mm (from midline) and H 7.9 mm (from dura; Pellegrino et al., 1979). The injection was

aimed at the rostral pole of the substantia nigra where the ascending nigrostriatal bundle converges. Accordingly, a maximum number of dopaminergic neurons can be lesioned by injecting into this particular site, resulting in a so-called near maximal lesion (Hudson et al., 1993). Following the injection, the needle was kept in place for another 10 min to allow diffusion of the toxin away from the injection site and to prevent back-flow.

2.2.3. Animal selection

Following surgery the animals were allowed to recover for at least 21 days before testing them in the rotameter. Selection of animals to be included in the studies was performed using the rotational response to apomorphine (0.25 mg/kg, s.c.) and only responders (> 100 net rotations) to this treatment were further used. The selected animals were used in subsequent experiments and the washout period between such experiments was at least 7 days. The animals were randomized for each new drug challenge.

2.2.4. Rotameter equipment

All animals were tested in automated rotameter cylinders (TSE, Bad Homburg, Germany) and the number of rotations (ipsilateral and contralateral) were automatically recorded.

2.2.5. Rotameter testing

2.2.5.1. MPEP alone. The animals were allowed to habituate to the rotameter bowls for 15 min. Subsequently, the animals received an injection of MPEP (doses: 7.5, 15 or 30 mg/kg, p.o.), the vehicle (methylcellulose (0.5%)) or (+)MK-801 (0.3 mg/kg, i.p.; a dose with a clear rotation response; Mele et al., 1998), i.e. the reference compound in these experiments (Clineschmidt et al., 1982). Following injection, the number of rotations was automatically recorded for 120 min.

2.2.5.2. Combined injections. Following an injection with either MPEP (doses: 7.5, 15 or 30 mg/kg, p.o.), the vehicle (methylcellulose (0.5%)) or (+)MK-801 (0.3 mg/kg, i.p.), the animals were placed in the rotameter bowls and allowed to habituate for 30 min. Subsequently, the animals received an injection of either apomorphine (0.25 mg/kg, s.c.), d-amphetamine (2.5 mg/kg, i.p.) or L-DOPA (25 mg/kg, i.p.). Following this injection, the number of rotations was automatically recorded for the next 90 to 240 min, depending upon the specific requirements of the experiment. An additional group was included in each experiment that was treated identically to the procedures outlined above but was injected with vehicle in order to determine the number of spontaneous rotations, i.e. the absolute control (abs).

2.2.6. Statistics

2.2.6.1. Rotarod experiment. Endurance performance on the rotarod was analysed using a two-factor repeated measures ANOVA (analysis of variance) with factors dose and time (repeated factor). Distinct time-points were compared using the Student's *t*-test (baclofen only).

2.2.6.2. Locomotor activity experiment. Activity counts were statistically evaluated using an ANOVA with factors dose and activity counts (horizontal and vertical) followed by Dunnett's test for multiple comparison of different dose levels with a control test (vehicle).

2.2.6.3. Rotation experiments. Statistical analysis was performed on the number of net rotations (ipsilateral-contralateral or vice versa) using an ANOVA followed by Dunnett's test, for multiple comparison of different dose levels (MPEP), or Student's *t*-test where appropriate ((+)MK-801; software: SYSTAT 8.0®).

3. Results

3.1. Rotarod experiments

MPEP induced no significant change in endurance performance when compared to controls on the accelerating rotarod at low dose range between 7.5 and 30 mg/kg, p.o. (ANOVA, $F = 0.096$, $p > 0.05$; Table 1a) nor at high-dose range between 100 and 300 mg/kg, p.o. (ANOVA, $F = 0.190$, $p > 0.05$; Table 1b) 1, 3, 6 or 24 h (high dose range only) after application. In contrast, baclofen, i.e. the positive standard, at a dose of 10 mg/kg, p.o. reduced the endurance performance to ±50% of controls ($P < 0.001$) 1 h after application. Endurance performance was again normalised 3 h after application.

3.2. Locomotor activity experiments

MPEP significantly reduced spontaneous horizontal ($F = 4.217$, $P < 0.01$) and vertical locomotor activity ($F = 5.512$, $P < 0.001$; Table 2). Although only the low dose of 7.5 mg/kg MPEP non-significantly increased locomotor activity, the high dose of 100 mg/kg MPEP significantly reduced horizontal locomotor activity (−38%; $P < 0.05$). Vertical activity was significantly reduced at doses of 30 and 100 mg/kg (−57%; $P < 0.05$ and −73%; $P < 0.001$, respectively).

3.3. 6-OHDA rotation experiments

3.3.1. MPEP-alone

MPEP increased the number of (ipsilateral) net rotations in a dose-dependent manner (ANOVA, $F = 5.940$, $P <$

Table 1
Accelerating rotarod

(a) Effect of MPEP (low doses)

Drug	Dose (mg/kg)	Endurance (s)				
		1 h	3 h	6 h	24 h	after application
Vehicle	0	254	267	280	nd	s
		14	13	11		S.E.M.
MPEP	7.5	270	258	257	nd	s
		15	12	15		S.E.M.
	15	256	262	279	nd	s
		19	17	11		S.E.M.
	30	244	280	287	nd	s
		16	11	6		S.E.M.

Mean (±S.E.M.) endurance performance on the accelerating rotating rotarod (4–40 rpm in 300 s) in rats ($n = 15$ per treatment group) at distinct time points (1, 3 and 6 h) after application of MPEP at doses of 7.5, 15 or 30 mg/kg, p.o. or vehicle (methylcellulose (0.5%)). nd = not determined

(b) Effect of MPEP (high doses)

Vehicle	0	244	274	281	283	s
		19	12	9	11	S.E.M.
MPEP	100	242	255	282	274	s
		19	20	12	13	S.E.M.
	200	249	256	268	242	s
		18	22	16	17	S.E.M.
	300	238	259	258	280	s
		25	22	13	14	S.E.M.
Baclofen	10	133[a]	268	296	288	s
		16	17	2	6	S.E.M.

Mean (±S.E.M.) endurance performance on the accelerating rotating rotarod (4–40 rpm in 300 s) in rats ($n = 12$ per treatment group) at distinct time points (1, 3, 6 and 24 h) after application of MPEP at doses of 100, 200, 300 mg/kg, p.o., baclofen 10 mg/kg, p.o. or vehicle (methylcellulose (0.5%)).

[a] $P < 0.001$ vs. vehicle (0 mg/kg).

0.002). Doses of 7.5 and 15 mg/kg (p.o.) may be considered as ineffective; in contrast, the dose of 30 mg/kg significantly increased the number of net rotations ($P < 0.001$, Fig. 1). Although statistically significant, the rotational behaviour induced by MPEP was completely different from that seen with (+)MK-801 (0.3 mg/kg, i.p.), i.e. the positive standard, and it was characterized by a slow and intermittent rotational response which tended to continue beyond the selected cut-off time of 120 min. Furthermore, quantitatively the increase in net rotations was small when compared to the effect of (+)MK-801 (0.3 mg/kg, i.p.). The latter increased net rotations six times more than that induced by 30 mg/kg MPEP (Fig. 1).

Table 2
Locomotor activity

Drug	Dose (mg/kg)	# Activity counts (mean ± S.E.M.)		
		horizontal	vertical	
MPEP	0	3555±409	628±95	counts
	3.75	3432±287	505±60	counts
	7.5	4482±508	699±115	counts
	15	3337±439	517±161	counts
	30	2816±283	270±44[a]	counts
	100	2197±233[a]	169±47[b]	counts

Mean (±S.E.M.) number of activity counts in the horizontal and vertical dimension in 120 min of registration following an injection with MPEP in the doses of 3.75, 7.5, 15, 30 or 100 mg/kg, p.o. or vehicle (methylcellulose (0.5%)). $n = 18$ per treatment group.

[a] $P < 0.05$.

[b] $P < 0.001$ vs. vehicle.

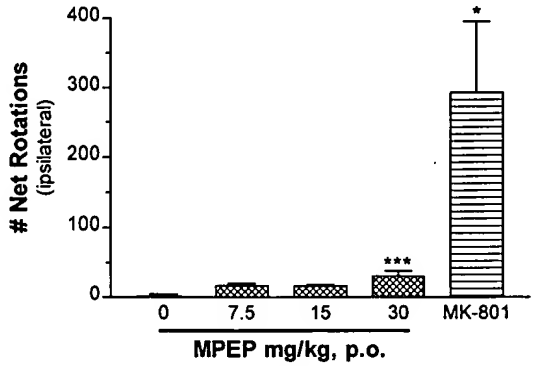

Fig. 1. MPEP-induced rotations: Bars represent the mean (±S.E.M.) number of net rotations as recorded during 120 min of registration following injection with MPEP at doses of 7.5, 15 or 30 mg/kg, p.o. ($n = 12$ per treatment group), the vehicle (methylcellulose (0.5%), $n = 11$) or (+)MK-801 (0.3 mg/kg, i.p., $n = 11$). * = $P < 0.05$, *** = $P < 0.001$ vs. vehicle (0 mg/kg).

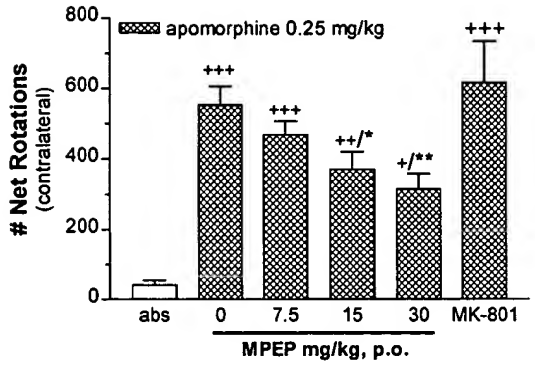

Fig. 2. Apomorphine-induced rotations: Bars represent the mean (±S.E.M.) number of net rotations in 90 min of registration following treatment with apomorphine (0.25 mg/kg, s.c.). The animals were pre-treated for 30 min with MPEP at doses of 7.5, 15 or 30 mg/kg, p.o. ($n = 12$ per treatment group), the vehicle (0 mg/kg; methylcellulose (0.5%), $n = 10$) or (+)MK-801 (0.3 mg/kg, i.p., $n = 11$). An additional group of animals was included that was treated with vehicle only, i.e. the absolute control (abs; $n = 7$). * = $P < 0.05$, ** = $P < 0.01$ vs. vehicle (0 mg/kg); + = $P < 0.05$, + + = $P < 0.01$, + + + = $P < 0.001$ vs. the absolute control (abs).

3.3.2. Combination MPEP and apomorphine

Apomorphine (0.25 mg/kg, s.c.) induced a significant increase ($P < 0.001$) in the number of net rotations when compared to the absolute control. MPEP decreased the number of apomorphine-induced net rotations in a dose-dependent manner (ANOVA, $F = 4.972$, $P < 0.005$). While 7.5 mg/kg of MPEP was ineffective, 15 and 30 mg/kg significantly reduced the number of net rotations ($P < 0.05$ and $P < 0.01$, respectively; Fig. 2). (+)MK-801 (0.3 mg/kg, i.p.) induced no statistically significant changes in apomorphine-induced rotations (Fig. 2).

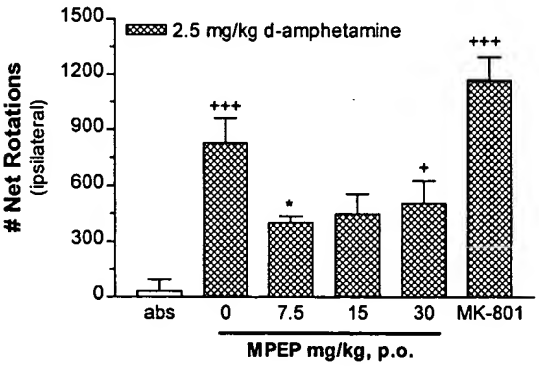

Fig. 3. D-Amphetamine-induced rotations: Bars represent the mean (±S.E.M.) number of net rotations in 180 min of registration following treatment with D-amphetamine (2.5 mg/kg, i.p.). The animals were either pre-treated for 30 min with MPEP at doses of 7.5, 15 or 30 mg/kg, p.o. ($n = 15$ per treatment group), the vehicle (0 mg/kg; methylcellulose (0.5%), $n = 15$) or (+)MK-801 (0.3 mg/kg, i.p., $n = 15$). An additional group of animals was treated with vehicle only, i.e. the absolute control (abs; $n = 12$). * = $P < 0.05$ vs. vehicle (0 mg/kg); + = $P < 0.05$, + + + = $P < 0.001$ vs. the absolute control (abs).

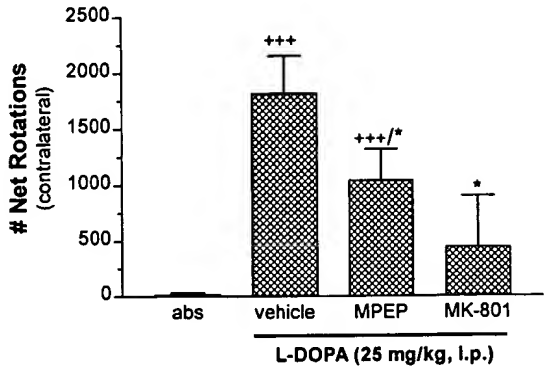

Fig. 4. L-DOPA-induced rotations: Bars represent the mean (±S.E.M.) number of net rotations in 240 min of registration following treatment with L-DOPA (25 mg/kg, i.p.). The animals were either pre-treated for 30 min with MPEP (30 mg/kg, p.o.; $n = 15$ per treatment group), the vehicle (0 mg/kg; methylcellulose (0.5%), $n = 13$) or (+) MK-801 (0.3 mg/kg, i.p., $n = 15$). An additional group of animals was treated with vehicle only, i.e. the absolute control (abs; $n = 5$). * = $P < 0.05$ vs. vehicle (0 mg/kg); + + + = $P < 0.001$ vs. the absolute control (abs).

3.3.3. Combination MPEP and D-amphetamine

D-Amphetamine (2.5 mg/kg, i.p.) induced a significant increase ($P < 0.001$) in the number of net rotations when compared to the absolute control. MPEP decreased the number of D-amphetamine-induced rotations (ANOVA, $F = 11.353$, $P < 0.001$; Fig. 3). While the dose of 7.5 mg/kg significantly reduced the number of net rotations, the effects of higher doses, i.e. 15 and 30 mg/kg did not reach the level of significance. In a separate experiment, the effect of lower doses (1 and 3 mg/kg) of MPEP was investigated and it was found that both doses were ineffective although the dose of 3 mg/kg tended to decrease the D-amphetamine-induced rotations (data not shown). (+)MK-801 (0.3 mg/kg, i.p.) potentiated the D-amphetamine-induced rotations; however, this effect did not reach significance ($P = 0.083$).

3.3.4. Combination MPEP and L-DOPA

L-DOPA (25 mg/kg, i.p.) induced a significant increase ($P < 0.001$) in the number of net rotations when compared to the absolute control (ANOVA, $F = 4.111$, $P < 0.05$). MPEP (30 mg/kg, p.o.) and (+)MK-801 (0.3 mg/kg, i.p.) significantly reduced L-DOPA-induced rotations ($P < 0.05$; Fig. 4).

4. Discussion

In the present study, four major findings were obtained. First, the rotarod experiments indicate that MPEP in the dose range between 7.5 and 300 mg/kg did not cause any deterioration of motor performance on the accelerating rotarod in rats. These data indicate that MPEP, even at fairly high doses of 100 mg/kg and up, and probably also other $mGlu_5$ receptor antagonists of the same chemical class, do not have serious deleterious effects on motor

behaviour in rats, such as ataxia or severe forms of sedation, rigidity or muscle relaxation.

Second, MPEP was found to inhibit spontaneous locomotor activity at doses of ≥ 30 mg/kg, i.e. at doses which induce turning behaviour in unilateral lesioned animals. Whether this effect reflects an influence on a clear motor component (however, see above) or an effect on premotor planning, is currently unknown and beyond the scope of the present study. Nevertheless, given the near maximal possible scores on the accelerating rotarod (see above), a non-motor component may be indicated.

Third, MPEP induced a dose-dependent ipsilateral rotational response in unilateral 6-OHDA lesioned rats. Although the effect was quite small, it was found consistently and has in the meantime been confirmed with other $mGlu_5$ receptor antagonists of the same chemical class (Spooren et al., unpublished observation). The fact that MPEP alone induced turning behaviour could favor the hypothesis of symptomatic benefit of $mGlu_5$ receptor antagonists in Parkinson's disease (see below). However, given the limited motor response following application of MPEP, these data may indicate that $mGlu_5$ receptor antagonists, if at all beneficial in Parkinson's disease (see below), may not be sufficiently effective to be used as a stand-alone symptomatic treatment to replace existing dopamine-based treatments. Furthermore, the finding that 30 mg/kg MPEP attenuated spontaneous locomotor activity in intact non-lesioned animals but increased activity in unilateral 6-OHDA-lesioned animal seems contradictive. Obviously, this underscores the need for a better understanding of the plasticity in this receptor in the basal forebrain and it may reflect the dynamics of $mGlu_5$ receptors in the diseased brain (see below).

Explaining the underlying mechanism of MPEP-induced rotations is mere speculation. However, it could be postulated that the effects induced by MPEP alone may reflect a direct interaction of dopamine and glutamate on striatal medium spiny neurons. As $mGlu_5$ receptors are highly expressed in striatal medium spiny neurons (Shigemoto et al., 1993; Romano et al., 1995), they may regulate the glutamatergic cortical-striatal input. Consequently, inhibition of this glutamatergic input is known to change the dopaminergic-glutamatergic balance in favor of dopamine (Amalric et al., 1994; Calabresi et al., 1997; Morari et al., 1998) and via such an action, MPEP may have induced the (weak) rotational response. Alternatively, inhibition of the excitatory subthalamic efferent pathways may equally well explain these findings. Clearly, the underlying mechanism remains to be further investigated in future studies.

Fourth, MPEP dose-dependently inhibited apomorphine-induced contralateral rotations. A straightforward subtraction of MPEP- and apomorphine-induced rotations cannot account for these findings: a dose of 15 mg/kg MPEP induced ± 20 ipsilateral rotations (90 min of registration) but MPEP inhibited ± 200 apomorphine-induced contralateral rotations (120 min of registration), i.e. approximately 10 times more. Given the fact that apomorphine mediates its effects post-synaptically and that both receptors, i.e. $mGlu_5$ and dopamine, are highly expressed in striatal medium spiny neurons, it is probable that these effects reflect an interaction of $mGlu_5$ and dopamine receptors on the level of the intra-cellular signaling pathways.

MPEP also inhibited D-amphetamine-induced ipsilateral rotations. Again, these data cannot be explained by a simple addition of their independent rotational effects since both compounds induce ipsilateral rotations. Although not completely without controversy (see: Hu et al., 1999), it has been suggested that the mixed groups I and II receptor agonist aminocyclopentane-1,3-dicarboxylic acid (ACPD) may increase dopamine release in freely moving animals (Bruton et al., 1999; cf. Bruton et al., 1996a,b). Further experiments indicated that especially the group I receptors may be responsible for this effect (Bruton et al., 1999). In addition, rotational behaviour is induced by direct injections of mixed groups I and II receptor agonists into the striatum (Smith and Beninger, 1996; also see: Sacaan et al., 1991, 1992). Taking these data together it could be hypothesized that the agonists of the $mGlu_5$ receptor may potentially increase dopamine release whereas $mGlu_5$ receptor antagonists in turn may inhibit dopamine release. Accordingly, MPEP may have inhibited D-amphetamine-induced dopamine release and causing a reduction in rotations. However, given the relative lack of $mGlu_5$ receptors on the pre-synaptic side, a post-synaptic mechanism, i.e. on the level of the intra-cellular signalling pathways as outlined above, may be more probable.

Finally, the effective dose of MPEP, i.e. 30 mg/kg, was chosen to evaluate its effect on L-DOPA-induced rotations. In line with the above discussed findings, MPEP significantly inhibited the L-DOPA-induced rotations. Again, a simple calculation of ipsilateral vs. contralateral rotations cannot explain the data; an interaction of the intra-cellular signalling pathways on the post-synaptic level may again seem likely (see discussion on apomorphine) although it is important to note that an interaction of different efferent pathways within or outside the basal ganglia can also explain these findings. Again mechanism of action awaits to be investigated in future studies.

The present findings indicate that any dopamine-mediated rotation response is inhibited by MPEP in the 6-OHDA lesioned rat. However, inhibition of dopamine-mediated behaviours is so far only seen in this lesion model since MPEP has been shown to have no effect on D-amphetamine-induced locomotor activity (Spooren et al., 2000) or on apomorphine-induced climbing in the intact mouse (Spooren et al., unpublished observation). Accordingly, these data suggest that in response to dopaminergic denervation the sensitivity of specific brain regions to mGluR5 antagonists may change markedly either within but also outside the affected brain regions.

The present study only partly confirmed the (+)MK-801-induced potentiation of dopamine-mediated responses or in the case of L-DOPA even opposite effects to those described in the literature were found (Carlsson and Carlsson, 1989; Klockgether and Turski, 1990; Morelli and Di Chiara, 1990; Morelli et al., 1992). It has to be noted that (+)MK-801 was used here as an effective reference compound and its interaction with either apomorphine or D-amphetamine was not per se the aim of investigation. Therefore, experimental conditions were not adapted according to anticipated results in a particular experiment but rather a fixed dose regimen was chosen. In the studies describing the potentiation of dopamine-mediated responses, the applied doses of (+)MK-801 were ineffective in inducing a rotational response by themselves (Carlsson and Carlsson, 1989; Klockgether and Turski, 1990; Morelli and Di Chiara, 1990; Morelli et al., 1992; Gossel et al., 1995). Since (very) low doses of (+)MK-801 potentiate dopamine-mediated responses in models of Parkinson's disease (Carlsson and Carlsson, 1989; Klockgether and Turski, 1990; Morelli and Di Chiara, 1990; Morelli et al., 1992), future studies might focus on effects of low-very low dosages of MPEP to indeed investigate a (+)MK-801-like action of MPEP.

It has been postulated that $mGlu_5$ receptors may be a novel drug target for the symptomatic treatment of Parkinson's disease (Nicoletti et al., 1997). Antagonists of mGlu receptors may reduce the activity of the overactive excitatory glutamatergic subthalamic-pallidal/nigral pathways that are thought to inhibit motor activity or provide symptomatic relief through other channels (Smith and Parent, 1988; Albin et al., 1989). Although the underlying mechanism is currently unknown, MPEP increased the number of net rotations as outlined above which may be an indication of treatment potential. Furthermore, MPEP has a relatively large safety margin since it did not induce any rotarod disturbance at doses of up to 300 mg/kg. However, a major drawback of $mGlu_5$ receptor antagonists for the treatment of Parkinson's disease is the fact that virtually any dopamine-mediated response in the 6-OHDA rat rotation model was shown to be inhibited by MPEP. In addition, MPEP at high doses inhibited spontaneous locomotor activity in rats. Accordingly, these data suggest that $mGlu_5$ receptor antagonists may counteract the symptomatic benefit provided by L-DOPA or by one of the direct dopamine receptor agonists currently on the market. Taking these present findings into consideration then it would become obvious that MPEP and potentially other $mGlu_5$ receptor antagonists are probably not appropriate drug candidates for the symptomatic treatment of Parkinson's disease.

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Chronic But Not Acute Treatment with a Metabotropic Glutamate 5 Receptor Antagonist Reverses the Akinetic Deficits in a Rat Model of Parkinsonism

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Metabotropic glutamate receptors (mGluRs) have recently been considered as potential pharmacological targets in the treatment of neurodegenerative disorders and particularly in parkinsonism. Within the basal ganglia, receptors of group I (mGluR1 and mGluR5) are widely expressed; the present study was thus aimed at blocking these receptors in a 6-hydroxydopamine (6-OHDA) model of Parkinson's disease in the rat. Considering the prominent expression of mGluR5, we have used the selective mGluR5 antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP) to target these receptors. In rats trained to quickly depress a lever after a visual cue, bilateral lesions of the dopaminergic nerve terminals in the striatum produced severe akinetic deficits, which were expressed by increases in delayed responses and reaction times. Acute MPEP injection (1.5, 3, and 6 mg/kg, i.p.) had no effect, whereas chronic administration, ineffective in a control group, significantly reversed the akinetic deficits. Alleviation of these deficits was seen after 1 week of treatment, and the preoperative performance was fully recovered after a 3 week treatment of MPEP at all doses. Chronic MPEP also induced ipsilateral rotation in the unilateral 6-OHDA circling model. However, no effect was seen of MPEP (1.5, 3, or 6 mg/kg, i.p.) on haloperidol-induced catalepsy (1 mg/kg, i.p.). Altogether, these results suggest a specific role of mGluRs in the regulation of extrapyramidal motor functions and a potential therapeutic value for mGluR5 antagonists in the treatment of Parkinson's disease.

Key words: basal ganglia; metabotropic receptor antagonist; 6-OHDA lesions; Parkinson's disease; reaction time task; glutamate; metabotropic receptors (mGluR5 subtype); MPEP; rat

Recent findings on the development of motor abnormalities in Parkinson's disease (PD) suggest a crucial involvement of increased glutamatergic activity in basal ganglia circuitry (Wichmann and DeLong, 1997). In experimental models of PD, reduction of excitatory amino acid transmission has thus been suggested to serve as a therapeutic alternative that may improve the motor symptoms in PD (Carlsson and Carlsson, 1989; Bergman et al., 1990; Schmidt et al., 1990; Amalric et al., 1995; Baunez et al., 1995; Rouse et al., 2000; Baron et al., 2002). In parkinsonian patients, surgical therapies have been successfully applied to improve symptomatology (Benabid et al., 1994; Limousin et al., 1995a,b). In addition to this surgical approach, non-invasive pharmacotherapies relying on drug discovery programs are actively pursued.

As a first approach, a number of studies showed that ionotropic glutamate receptor antagonists of the NMDA subtypes could counteract parkinsonian symptoms or act in synergy with L-3,4-dihydroxyphenylalanine (L-DOPA) in animal models of PD (Greenamyre and O'Brien, 1991; Schmidt et al., 1992; Ossowska, 1994; Danysz et al., 1997; Starr et al., 1997). The alleviation of parkinsonian motor signs was often limited, however, because of the occurrence of uncontrolled side effects at higher dose regimens (hallucinations, cognitive perturbations, postural imbalance) (Amalric et al., 1995; Andine et al., 1999). The narrow window between symptomatic relief and side effects with these NMDA receptor antagonists gave rise to the hypothesis that a modulatory action on glutamate transmission would avoid some of these undesirable side effects.

Recent emphasis has been placed on metabotropic glutamate receptors (mGluRs) in the treatment of neurodegenerative disorders. On the basis of primary sequence, second messenger coupling, and pharmacological profiles, mGluRs can be classified into three subgroups: group I (mGluR1 and mGluR5), group II (mGluR2 and mGluR3), and group III (mGluR4, 6, 7, and 8) (for review, see Conn and Pin, 1997). The expression of mGlu5 receptors in the basal ganglia suggests that this receptor subtype might be an interesting target in the treatment of PD. Indeed, mGlu5 receptors have been implicated as major players in the excitatory drive to the subthalamic nucleus from glutamatergic afferents (Awad et al., 2000). The recent identification of a selective and systemically active ligand for the mGluR5 subtype, i.e., 2-methyl-6-(phenylethynyl)-pyridine (MPEP) (Gasparini et al., 1999), allowed us to evaluate the potential therapeutic benefit in animal models for nervous system disorders (Spooren et al., 2001). The present study therefore tested the effects of MPEP administration in a rat model of PD induced by bilateral 6-OHDA lesions in the striatum. It was shown previously that this model produces profound deficits in a reaction time task (Amalric and Koob, 1987; Amalric et al., 1995; Baunez et al., 1995). The

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effects of acute or chronic application of MPEP were thus tested in this model of akinesia and in additional models of PD: the unilateral 6-OHDA rotation and the haloperidol-induced catalepsy.

MATERIALS AND METHODS

Experiment 1: reaction time task

Animals

Male Wistar rats (n = 85; Iffa Credo, Lyon, France), weighing 110–120 gm at the beginning of the experiment, were housed in groups of two per cage and maintained in temperature-controlled conditions with a 12 hr light/dark cycle (7 A.M.-7 P.M., lights off). Their food supply was restricted to 15–17 gm/d per rat to keep them at 80% of the free-feeding weight of control animals. Water was provided *ad libitum*.

All procedures were conducted in accordance with the requirements of the French " Ministère de l'agriculture et de la pêche" Décret no. 87–848, October 19, 1987.

Behavioral procedure

Eight operant boxes (Campden Instruments, Cambridge, UK) were used for the reaction time (RT) task. Each box was equipped with a lever, a food magazine, and a cue light (a 2.8 W bulb) located above the lever corresponding to the conditioning stimulus (CS). The lever required a force of 0.8 N for switch closure. A house light located on the ceiling was turned on at the beginning of the testing session. Each box was placed in a wooden sound-attenuating cabinet that was ventilated by a low-level noise fan. Rats were trained to depress the lever and wait for the onset of the visual trigger stimulus presented after four randomly and equiprobably generated intervals (0.5, 0.75, 1.00, or 1.25 sec.). To be rewarded by a food pellet (45 mg; Phymep, Paris, France), the rat was required to release the lever with a RT below 600 msec. The RT was measured in milliseconds from the onset of the stimulus to the lever release. Each daily session ended after 100 trials. Performance was evaluated by recording the number of correct and incorrect (nonrewarded) responses as either "premature," corresponding to early withdrawal of the lever (before the onset of the CS), or "delayed," when the lever was released with RT above 600 msec. After training, rats were tested for 6 consecutive days in the RT task from preoperative baseline values before surgery. After a 7 d postoperative recovery period, they were tested again for 24 sessions up to 32 d.

Dopamine lesion

The animals were anesthetized by an intramuscular injection of xylazine (15 mg/kg) and ketamine (100 mg/kg) and placed in a stereotaxic instrument (David Kopf Instruments) with the incisor bar positioned −3.0 mm under the interaural line for surgical procedures based on coordinates of Paxinos and Watson (1986). Lesioned animals received a bilateral injection of 6-OHDA hydrochloride (Sigma Aldrich, Lyon, France) (4 μg/μl, 3 μl per side) in the striatum at the following coordinates: anteroposterior (AP) +0.2 mm, lateral (L) ±3.5 mm, dorsoventral (DV) −4.8 mm (from skull) according to bregma. The sham control group received the vehicle alone (ascorbate solution, 0.1 mg/ml) in the dorsal striatum. The infusion was made with a micropump over 9 min using a 10 μl Hamilton microsyringe, connected by a Tygon tubing fitting to the 30 gauge stainless steel injector needles. A 1 week recovery period was allowed before the animals were again tested daily on the behavioral task.

Drugs

6-OHDA (Sigma Aldrich) was dissolved in ascorbic acid solution (0.1 mg/ml in 0.9% saline) to prevent oxidation. MPEP hydrochloride (Novartis, Basel, Switzerland) was dissolved in distilled water and injected intraperitoneally in a volume of 1 ml/kg.

Experimental procedure

Acute MPEP treatment. Twenty-four rats were used to test the effects of an acute injection of MPEP. Control rats ("sham"; n = 9) and dopamine (DA)-depleted rats (6-OHDA; n = 15) were tested on the RT task. The effects of the lesion were tested between days 9 and 14 after surgery. Each group (sham vs 6-OHDA) was then divided into three different groups receiving three doses of MPEP (0.3, 1, or 3 mg/kg) in a different order of injection over 3 weeks following a Latin-square design. Injections were performed once a week over 3 weeks.

Chronic MPEP treatment. The effects of 6-OHDA lesion were tested between postoperative days 9 and 14. MPEP was then injected intraperitoneally for 3 weeks (days 15–31 after surgery), and the animals were immediately tested in the RT task. The animals were divided into four subgroups depending on the dose of MPEP (MPEP 0, n = 10; MPEP 1.5, n = 7; MPEP 3, n = 10; MPEP 6, n = 10). The effects of MPEP chronic treatment were tested further in a control group of animals (sham operated) under the same experimental conditions. MPEP was chronically injected intraperitoneally for 3 weeks at the same doses (i.e., 0, 1.5, 3, and 6 mg/kg; n = 6 for each group, except n = 8 for the 0 group) between postoperative days 9 and 31.

Statistical analysis

The effects of dopamine depletion and MPEP treatments on RT performance were evaluated on each variable (i.e., number of correct, premature, and delayed responses and RTs) averaged across each session. For each variable, the data were submitted to a mixed design ANOVA with different subgroups ("6-OHDA" vs "MPEP1.5" vs "MPEP3" vs "MPEP6") and different orders (for the acute experiment) as the between-subject factor, the sessions (6 before surgery, 6 after surgery, and 18 with chronic treatment or the acute treatment sessions) as the within-subject factors, as appropriate. *Post hoc* multiple comparisons between groups were made using simple main effects analysis and Fisher test, as appropriate.

To detect whether rats had a preparatory motor strategy to perform the conditioned reaction time task (using the visual stimulus occurrence, i.e., the shorter RTs are associated with the longer delay), RTs were plotted as a function of the intervals preceding the CS at preoperative day 2 and postoperative days 12 and 31. The ANOVA involved two within-subjects factor: the four various intervals and the preoperative and postoperative sessions (Statview 5.0 program, Abacus concept).

Histology

At the end of the experiment, animals were killed by decapitation. The brains were then removed and frozen to −80°C. Coronal 10 μm tissue sections were cut at −20°C using a microtome cryostat (Leica CM3050) at the level of the striatum.

The binding of [^{3}H]-mazindol to dopamine uptake sites in the striatum was measured according to the procedure described by Javitch et al. (1985). Briefly, sections were air dried and rinsed for 5 min at 4°C in 50 mM Tris buffer with 120 mM NaCl and 5 mM KCl. They were then incubated for 40 min with 15 nM [^{3}H]-mazindol (NEN DuPont; specific activity 17 Ci/mM) in 50 mM Tris buffer containing 300 mM NaCl and 5 mM KCl added with 0.3 mM desipramine to block the noradrenalin transporter. Nonspecific binding was determined by incubating some sections in the same solution plus 30 mM benztropine. Sections were rinsed twice for 3 min in the incubation medium without mazindol and for 10 sec in distilled water and were air dried. Autoradiographs were generated by apposing the sections to ^{3}H-sensitive screen (Raytest) for 7 d and were further quantified with a β imager (Fuji-Bas 5000).

Experiment 2: turning behavior

Animals

Male Sprague Dawley rats (Iffa Credo, Les Oncins, France; n = 120) weighing 250–280 gm at the time of surgery (see below) were used. The animals were housed four per cage in a temperature-controlled room (22 ± 1°C) under artificial illumination (6 A.M.-6 P.M., lights on) with *ad libitum* access to water and food (Ecosan, Eberle Nafag AG, Gossau, Switzerland).

Surgery

Before surgery, all animals received an injection of desipramine hydrochloride (30 mg/kg, i.p.; USPC Inc., Rockville, MD) to protect noradrenergic cells. One hour later the animals received an injection of pentobarbital (55 mg/kg, i.p.; Vetanarcol, Veterinaria AG, Zurich, Switzerland) and were subsequently placed (under deep anesthesia) in a stereotaxic apparatus. A unilateral lesion was made by injecting 9 μg 6-OHDA (hydrobromide; Fluka Chemie AG, Buchs, Switzerland) in 0.7 μl ascorbic acid solution (dilution 1 mg/ml) over 10 min into the left medial forebrain bundle [coordinates: AP 3.6 mm (from bregma), L 1.1 mm (from midline), and DV −7.9 mm (from dura); Pellegrino et al. (1997)]. The control group received the ascorbic solution at the same

coordinates. The injection was aimed at the rostral pole of the substantia nigra where the ascending nigrostriatal bundle converges to produce a so-called near-maximal lesion (Spooren et al., 1999). After the injection, the needle was kept in place for another 10 min to allow diffusion of the toxin away from the injection site and to prevent backflow.

After surgery, the animals were allowed to recover for at least 21 d before they were tested in the rotameter. Selection of animals to be included in the studies was performed using the rotational response to apomorphine (0.25 mg/kg, s.c.), and only responders (>100 net rotations) to this treatment were used.

Procedures for rotameter testing

All animals were tested in automated rotameter cylinders (TSE, Bad Homburg, Germany), and the number of rotations (ipsilateral and contralateral) was recorded automatically.

The animals (n = 16 for each treatment group) received one injection with MPEP (doses: 7.5 or 30 mg/kg, p.o.) or vehicle (methylcellulose, 0.5%) per day for 7 d. Considering the bioavailability of MPEP by oral administration, the doses of MPEP injected by mouth were comparable to those administered intraperitoneally in the RT task. They were chosen according to earlier studies examining the effects of acute MPEP on rotation (Spooren et al., 1999). The turning behavior after the first and seventh injection were recorded automatically (see above) in the rotameter. The effects of injections two to six were not recorded, and after the injection the animals immediately returned to their home cages.

Statistical analysis

The number of net rotations (i.e., the number of contralateral rotations minus the number of ipsilateral rotations) per treatment was statistically evaluated by means of a repeated measures ANOVA with one factor between groups (dose: 0, 7.5, or 30 mg/kg, p.o.) and two factors within groups (repeated), i.e., tests days (day 1 or 7) and time points (10, 20, 30, 40, 50, 60, 70, 80, and 90 min after injection). The number of net rotations at distinct time points between treatment groups [vehicle vs MPEP (7.5 or 30 mg/kg, p.o.) or within treatment groups (one injection vs seven injections)] were compared using an unpaired and paired t test, respectively; $p < 0.05$ was considered as statistically significant (Software: Systat 10.0).

Experiment 3: catalepsy

To compare the effects of acute and chronic administration of MPEP on haloperidol-induced catalepsy, the animals were tested in the horizontal bar test as follows. Each animal was gently placed with its forepaws on a metal rod suspended 9 cm above the floor, and the time elapsing before it climbed down from the bar was recorded in seconds. The mixed D1/D2 dopaminergic receptor antagonist haloperidol (Haldol injectable solution; Janssen, Boulogne, France) was dissolved in physiological 0.9% saline solution and injected systemically at a dose of 1 mg/kg. In the two experimental procedures (acute vs chronic) haloperidol (or its solvent) was injected 20 min before MPEP injection, and catalepsy was measured every 20 min during the 3 hr testing.

Acute MPEP treatment on haloperidol-induced catalepsy. Twenty-one rats were used to test the effects of an acute injection of MPEP on the cataleptogenic effects of haloperidol. The animals were divided into three groups depending on the dose of MPEP (MPEP 0, n = 7; MPEP 3, n = 7; MPEP 6, n = 7). A group of eight control rats received haloperidol vehicle 20 min before MPEP solvent injection.

Chronic MPEP treatment on haloperidol-induced catalepsy. Thirty-two rats were used to test the effects of chronic injection of MPEP on the cataleptogenic effects of haloperidol. The rats were divided into four groups depending on the dose of MPEP (MPEP 0, n = 8; MPEP 1.5, n = 8; MPEP 3, n = 8; MPEP 6, n = 8). Each animal received a 3 week treatment with MPEP before receiving the haloperidol injection.

Statistical analysis

Catalepsy data were analyzed nonparametrically by performing a multiple Kruskal-Wallis "H" test, and the median latency was calculated for each dose and for each 20 min period. Individual comparisons were performed using the nonparametric Mann–Whitney U test.

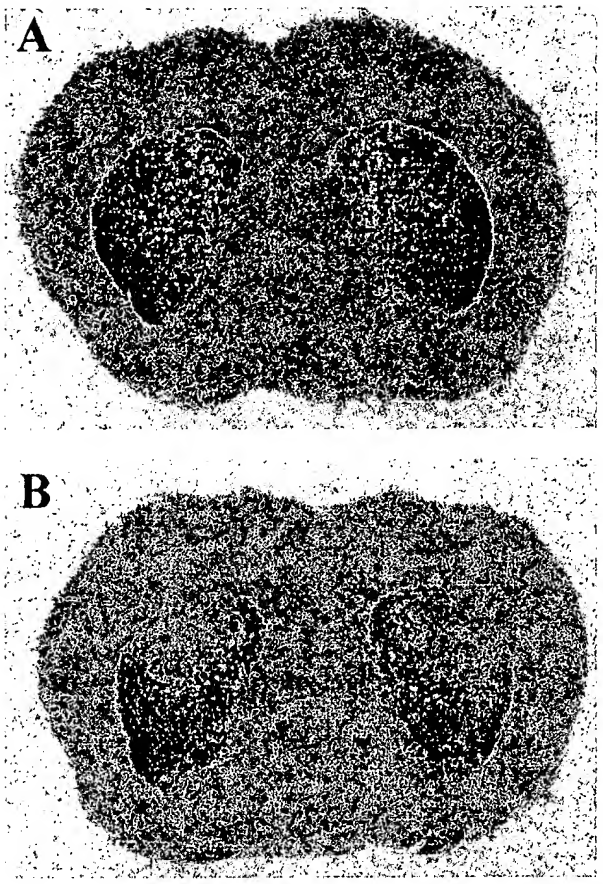

Figure 1. Binding of [^{3}H]-mazindol to dopamine uptake sites at the striatum level. Photomicrographs comparing the level of [^{3}H]-mazindol labeling in striatal sections from a control animal (A) and a bilaterally lesioned animal (B). The lack of mazindol binding in B shows the restricted size of the 6-OHDA lesion in the dorsal striatum as compared with sham animals.

RESULTS

Experiment 1: reaction time task

Histology

The binding of [^{3}H]-mazindol to dopamine uptake sites in the striatum as determined on coronal sections was used to delineate the extent of dopamine depletion induced by the bilateral striatal 6-OHDA injections (Fig. 1). It was found that the dopamine lesions were consistently restricted to the dorsolateral part of the striatum at the rostral level and extended more ventrally at the more caudal levels (end of the anterior commissure).

6-OHDA lesion effect on correct and incorrect responses

The effects induced by 6-OHDA infusion into the dorsal part of the striatum were analyzed on postoperative days 9–14. As illustrated in Table 1 and Figure 2, the number of correct responses was markedly reduced as compared with the preoperative levels within each of the five lesioned groups (i.e., acute, 6-OHDA, MPEP1.5, MPEP3, and MPEP6 groups). 6-OHDA lesions resulted in a significant decrease in the correct responses whatever the group tested ($p < 0.05$; paired t test after significant ANOVA, $F_{(4,56)} = 21.60$, $F_{(4,36)} = 3.30$, $F_{(4,24)} = 4.82$, $F_{(4,36)} = 12.19$ and 8.25). This effect was also found to be significantly different from correct responses of the sham-operated group

Table 1. Effects of acute MPEP treatment on RT task performance

			MPEP (mg/kg)		
	Pre	Post	0.3	1	3
Correct					
Sham (n = 9)	70 ± 3.2	71 ± 2.6	72 ± 3.0	71 ± 3.9	63 ± 4.5
6-OHDA (n = 15)	68 ± 3.2	41 ± 4.5[a,b]	42 ± 4.2[a,b]	42 ± 4.7[a,b]	34 ± 4.8[a,b]
Premature					
Sham	25 ± 3.4	22 ± 2.6	21 ± 2.8	23 ± 3.4	30 ± 3.8
6-OHDA	27 ± 2.8	44 ± 4.7[a,b]	44 ± 4.5[a,b]	44 ± 5.6[a,b]	48 ± 5.1[a,b]
Delayed					
Sham	6 ± 1.0	7 ± 1.8	7 ± 1.7	6 ± 1.0	7 ± 1.9
6-OHDA	5 ± 0.9	15 ± 2.5[a,b]	14 ± 2.5[a]	14 ± 2.3[a,b]	14 ± 2.8[a]

The values correspond to the mean number of correct, premature, and delayed responses during different sessions: one preoperative (pre); one postoperative (post) at day 12 after the lesion; and on the session recorded immediately after MPEP injections at various doses (0.3, 1, and 3 mg/kg), in sham animals and 6-OHDA-lesioned rats.

[a] $p < 0.05$, paired t test, as compared with sham group after significant ANOVA.

[b] $p < 0.05$, paired t test, as compared with preoperative performance after significant ANOVA.

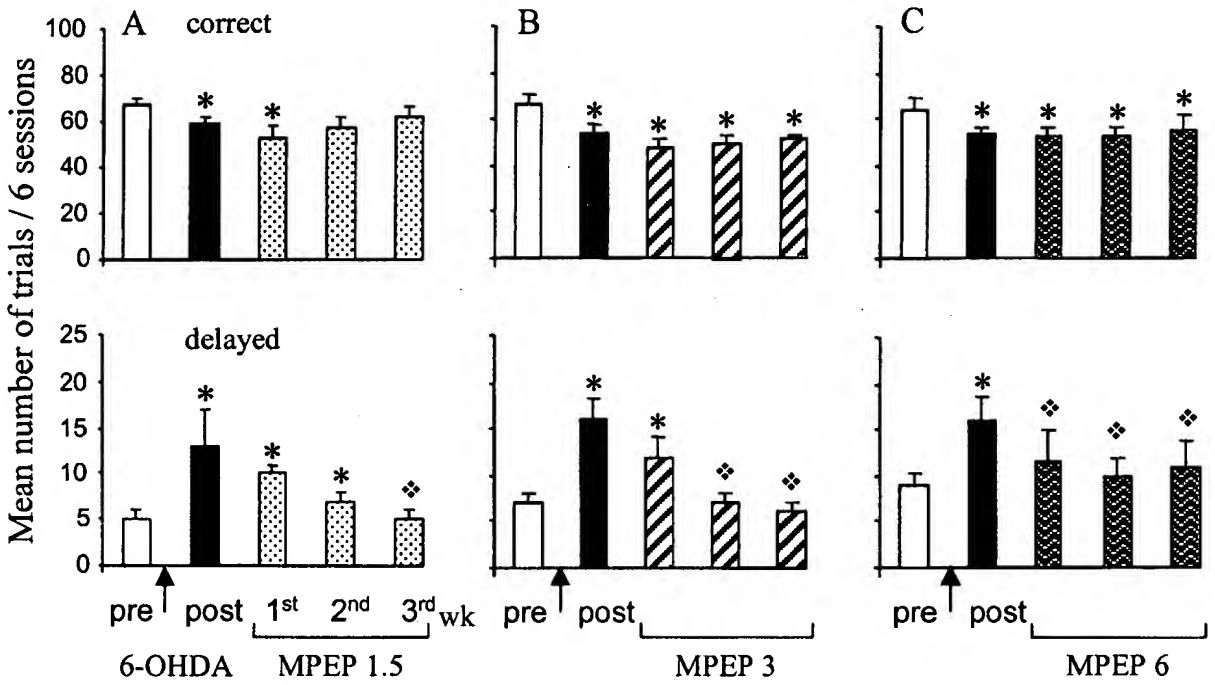

Figure 2. Effects of chronic MPEP treatment on correct and delayed performance after 6-OHDA lesion. The *vertical axes* give the mean number of trials ± SEM per block of six sessions for the two variables measured (*correct* and *delayed* responses). The effects are measured during various blocks of six sessions corresponding to the following: one block before the surgery (*pre*), one from day 9–14 after lesion (*post*), and three blocks during MPEP chronic treatment from days 15–20, 21–26, and 27–32 after lesion, respectively, of the first, second, and third week. The effects induced by the different doses of MPEP [1.5 mg/kg (*A*), 3 mg/kg (*B*), and 6 mg/kg (*C*)] are compared with the preoperative and postoperative levels. *, Significant difference from preoperative performance ($p < 0.05$; paired t test after significant ANOVA). ❖, Significant difference from postoperative performance ($p < 0.05$ paired t test after significant ANOVA).

($p < 0.05$; Newman–Keuls test) and greater in magnitude in the "acute" 6-OHDA group than the "chronic" group because of a significant increase in premature responding (Table 1). This was previously found to result from slightly more ventral diffusion of the 6-OHDA neurotoxin in the striatum (Amalric et al., 1995). The effects of 6-OHDA lesions were long lasting, and no recovery of baseline performance was observed 32 d after surgery in the 6-OHDA group (data not shown).

The decreased number of correct responses was mainly caused by a significant increase of delayed responses in all the lesioned groups ($p < 0.05$; paired t test after significant respective ANOVA, $F_{(4,56)} = 5.59$, $F_{(4,36)} = 7.02$, $F_{(4,24)} = 4.78$, $F_{(4,36)} = 10.43$ and 8.03). This effect was also significant in comparison with sham control group performance ($p < 0.05$; Newman–Keuls test).

Reaction time and motor readiness

In addition, the dopamine depletion in the dorsal striatum significantly increased RTs when compared with preoperative values ($p < 0.05$; paired t test) (see Fig. 4*A*). At day 32 after lesion, RTs averaged a value of 422 ± 25.25 msec in comparison with 330 ± 18 msec on day 3 preceding the lesion ($p < 0.05$; paired t test). We also investigated whether 6-OHDA lesion disrupted the re-

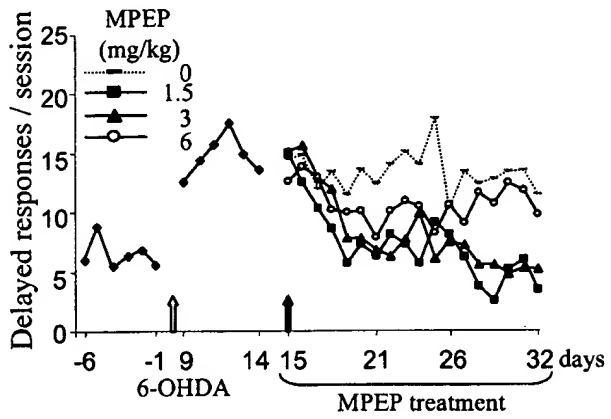

Figure 3. Chronic MPEP treatment on delayed responses over time in the RT task. The mean number of delayed responses per session is illustrated for the 6 preoperative sessions (days −6 to −1), the 6 postoperative sessions (days 9–14), and the 18 sessions (days 15–32) of chronic treatment with distilled water, MPEP 1.5 (n = 7), MPEP 3 (n = 10), or MPEP 6 (n = 10). Animals were tested every day in a 100-trial session.

sponse preparatory processes, also termed "motor readiness." It was found that RTs significantly decreased as a function of the variable intervals (significant "interval" effect ANOVA, $F_{(3,24)}$ = 7.80 for preoperative performance), suggesting optimal motor preparatory processes. This effect on motor readiness remained significant whatever the postoperative day (ANOVA, $F_{(3,24)}$ = 7.61 and 3.03 at 12 and 32 performance days after lesion, respectively).

Acute MPEP treatment on correct and incorrect responses

No significant effect of the order of injection of the various doses tested according to the Latin-square design was found for any variable ("order" effect, all $F_{(2,18)}$ <0.77; interaction order × "dose," all $F_{(6,54)}$ < 1.18 for all measures). As shown in Table 1, the acute treatment with various doses of MPEP (0.3, 1, and 3 mg/kg) had no effect on the behavioral performance after the 6-OHDA lesion; the number of correct responses remained significantly lower than the preoperative level ($p < 0.05$; paired t test after significant ANOVA, $F_{(4,56)}$ = 21.61), whereas the number of delayed and premature responses remained significantly higher ($p < 0.05$; paired t test after significant ANOVA, $F_{(4,56)}$ = 5.59; and ANOVA, $F_{(4,56)}$ = 6.29, respectively).

Chronic MPEP treatment on correct and incorrect responses

6-OHDA. Chronic treatment with MPEP normalized the number of delayed responses of 6-OHDA-lesioned rats at all doses tested, as shown in Figure 2. One week of treatment with 6 mg/kg MPEP was sufficient to produce this beneficial effect ($p < 0.05$; paired t test comparing the 3 weeks of treatment with post-lesion level), whereas 2–3 weeks of treatment at a lower dose were required to produce the same effect (3 and 1.5 mg/kg). At the end of the chronic treatment with MPEP, all selected doses had induced a full recovery of these responses in comparison with postoperative level of performance ($p < 0.05$; paired t test; after significant treatment effect ANOVA, $F_{(4,36)}$ = 8.03, $F_{(4,36)}$ = 10.43, $F_{(4,24)}$ = 4.78 for 6, 3, and 1.5 mg/kg, respectively). The time effect of this recovery is illustrated as day-by-day performance in Figure 3.

MPEP at a dose of 1.5 mg/kg normalized the number of correct responses at the second week of treatment and this effect remained stable until the end of the experiment (no significant difference between pretreatment and second and third week of treatment). This effect was not observed after MPEP treatment at the doses of 3 and 6 mg/kg. MPEP was found to induce an increase in premature responding that prevented the normalization of correct responses (Table 2) ($p < 0.05$; paired t test; after significant "treatment" effect on premature responses ANOVA, $F_{(4,36)}$ = 12.65 and 4.23, respectively).

Control group. Chronic treatment with MPEP at the same doses in a control group of animals (i.e., no lesion), trained previously in the RT task, did not significantly modify the number of correct, premature, or delayed responses, except in the group treated with 3 mg/kg MPEP. Chronic treatment with 3 mg/kg MPEP was found to transiently decrease the number of correct responses (ANOVA, $F_{(4,20)}$ = 3.21; $p < 0.05$) associated with a nonsignificant increase in premature responding (Table 3). However, no reduction of delayed responses was observed at any dose tested in the control animals, suggesting a selective effect of the compound in 6-OHDA-lesioned animals in this parameter. No ataxia or any debilitating effects were observed on behavior whatever the dose of MPEP used.

Reaction time and motor readiness. The increase in RTs induced by a DA depletion in the dorsal striatum was totally reversed at day 32 after lesion in animals treated with either 1.5 or 3 mg/kg (no significant difference when compared with preoperative performance; $p > 0.05$; paired t test) (Fig. 4*B*,*C*). The motor readiness effect was not affected by chronic treatment with either 1.5 or 3 mg/kg MPEP [significant interval effect ANOVA, $F_{(3,18)}$ = 5.69 and $F_{(3,24)}$ = 7.14 for day 32 after lesion performance in the two MPEP groups (1.5 and 3 mg/kg, respectively)].

Experiment 2: turning behavior

The ANOVA indicated statistical significance for factors dose ($F_{(2,48)}$ = 8.81), test day ($F_{(1,48)}$ = 7.19), time ($F_{(3,384)}$ = 16.22), and the interaction test × time point ($F_{(8,384)}$ = 8.48) using the number of net rotations (contralateral − ipsilateral rotations) as dependent variable.

Vehicle treatment

At no time point were statistically significant differences found in the number of net rotations within vehicle-treated animals after the chronic (seven times) treatment in comparison with the first (acute) vehicle injection (Fig. 5).

Vehicle versus MPEP

After the acute and chronic vehicle treatment, the animals exhibited a preference for spontaneous contralateral rotations during the registration period of 90 min. In contrast, after the acute and chronic treatment with MPEP (one time and seven times 7.5 or 30 mg/kg, p.o.), an ipsilateral rotation preference was found that resulted in statistically significant increases in the number of ipsilateral net rotations at distinct time points as compared with acute and chronic vehicle controls (Fig. 5).

MPEP treatment (acute versus chronic)

Chronic (seven times) application of MPEP (7.5 and 30 mg/kg, p.o.) induced a significant increase in the number of (ipsilateral preference) net rotations 30 and 40 min after the injection as compared with the acute treatment with these doses of MPEP.

Experiment 3: catalepsy

Haloperidol (1 mg/kg) produced a profound increase in catalepsy as shown by a progressive increase in the median latency to step down the rod over time as compared with controls ($p < 0.05$;

Table 2. Effects of chronic MPEP treatment on premature performance after 6-OHDA lesion

MPEP (mg/kg)	Pre	Post	First week	Second week	Third week
0	28 ± 3.2	28 ± 3.2	28 ± 3.1	27 ± 2.5	29 ± 2.4
1.5	28 ± 4.0	30 ± 5.0	37 ± 4.0	35 ± 6.0	34 ± 5.0
3	27 ± 2.5	29 ± 3.6	41 ± 3.5[a,b]	41 ± 2.6[a,b]	44 ± 1.6[a,b]
6	26 ± 3.0	29 ± 3.0	39 ± 4.0[a,b]	38 ± 4.0[a,b]	33 ± 3.0

Values correspond to the mean number of premature responses ± SEM per six sessions during the various blocks of the experiment. The first block (pre) represents the six sessions preceding the surgery, the second (post) corresponds to the six sessions after lesion, the three others correspond to the 3 weeks of MPEP treatment (first, second, and third weeks).

[a]Significant difference from preoperative performance ($p < 0.05$ paired t test after significant ANOVA).

[b]Significant difference from postoperative performance ($p < 0.05$ paired t test after significant ANOVA).

Table 3. Effects of chronic MPEP treatment on RT task performance of control animals (no lesion)

MPEP (mg/kg)	Pre	Post	First week	Second week	Third week
Correct					
0	67 ± 4.3	68 ± 2.0	70 ± 2.6	68 ± 4.7	69 ± 4.2
1.5	69 ± 4.3	64 ± 5.6	62 ± 5.7	64 ± 6.0	62 ± 3.0
3	67 ± 4.8	65 ± 2.7	58 ± 4.0[a]	59 ± 5.0[a]	58 ± 6.0
6	68 ± 3.9	63 ± 2.0	61 ± 3.9	59 ± 6.1	56 ± 3.0
Premature					
0	28 ± 4.7	27 ± 1.7	24 ± 1.7	26 ± 4.8	25 ± 3.9
1.5	25 ± 4.3	30 ± 5.8	30 ± 5.6	28 ± 6.0	31 ± 4.0
3	27 ± 5.2	28 ± 2.1	36 ± 4.7	36 ± 5.0	37 ± 6.0
6	26 ± 3.7	31 ± 1.9	30 ± 3.7	35 ± 6.1	35 ± 5.0
Delayed					
0	5 ± 1.4	5 ± 1.1	6 ± 1.2	6 ± 1.2	6 ± 1.6
1.5	6 ± 1.2	7 ± 1.7	9 ± 1.5	8 ± 2.0	7 ± 1.0
3	6 ± 1.2	7 ± 2.7	6 ± 1.7	5 ± 1.0	5 ± 2.0
6	7 ± 1.0	6 ± 2.0	9 ± 2.4	6 ± 2.5	5 ± 2.0

Values correspond to the mean number of correct, premature, and delayed responses during the different sessions: preoperative (pre), postoperative (post), and the 3 weeks of treatment at the various doses of MPEP (0, 1.5, 3, and 6 mg/kg).

[a]Significant difference from postoperative performance ($p < 0.05$ paired t test after significant ANOVA).

Mann–Whitney U test after Kruskal–Wallis test; $H = 80.65$ and 103.68 for the first and second experiment, respectively) (Fig. 6*A*,*B*). Catalepsy induced by haloperidol was not significantly antagonized by the acute injection of MPEP (3 and 6 mg/kg, i.p.; $p > 0.05$), although 3 mg/kg MPEP showed a trend toward reversal of catalepsy ($p = 0.07$) 150 min after haloperidol injection (Fig. 6*A*). In contrast, 6 mg/kg MPEP did not modify the cataleptic state of the rats. When injected chronically for 3 weeks at whatever the dose tested (1.5, 3, and 6 mg/kg), MPEP did not significantly influence haloperidol-induced catalepsy (Fig. 6*B*).

DISCUSSION

The present results demonstrate that chronic but not acute treatment with MPEP, i.e., a selective mGluR5 antagonist, has remarkable beneficial effects on the expression of certain deficits in a reaction time task induced by bilateral injections of 6-OHDA into the striatum. In this rodent model of Parkinson's disease, the dopaminergic lesions impaired the performance by increasing the number of delayed responses and lengthening RTs, which suggests a deficit in motor planning. These akinetic deficits were fully reversed by MPEP treatment at all doses tested (1.5, 3, and 6 mg/kg). Animals treated with the lowest doses of MPEP were able to reach all prelesion levels of performance within 3 weeks. Interestingly, acute injections of MPEP in 6-OHDA animals or chronic treatment at the same doses in nonlesioned animals did not modify the performance. Furthermore, it is shown that chronic treatment with MPEP also significantly increases ipsilateral rotations in the classical unilateral 6-OHDA lesion circling model. In contrast, in the model of PD involving dopamine receptor antagonist administration (i.e., the catalepsy test), chronic treatment with MPEP was ineffective.

Recent evidence suggests that the neurodegeneration of the dopamine neurons of the substantia nigra pars compacta, eliciting the motor symptoms of PD, results in an increase in glutamatergic activity at the striatal and subthalamic nucleus (STN) levels of the basal ganglia. Ultimately, the increase in glutamate activity of the STN is responsible for overactivity in the basal ganglia output structures [i.e., internal segment of the globus pallidus (GPi) and the substantia nigra pars reticulata (SNr)] that are also directly under the control of the striatum (Rouse et al., 2000). In the primate 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine model of PD, the expression of the mRNA encoding for cytochrome oxidase subunit I, a molecular marker for functional neuronal activity, was found to be enhanced in the STN and in the SNr/GPi (Vila et al., 1997). Therefore, several attempts have been made to reduce STN overactivity by using pharmacological as well as surgical tools. In parkinsonian patients, high-frequency stimulation of the STN, which blocks STN neuronal activity (Benazzouz et al., 2000; Beurrier et al., 2001), alleviates the motor symptoms

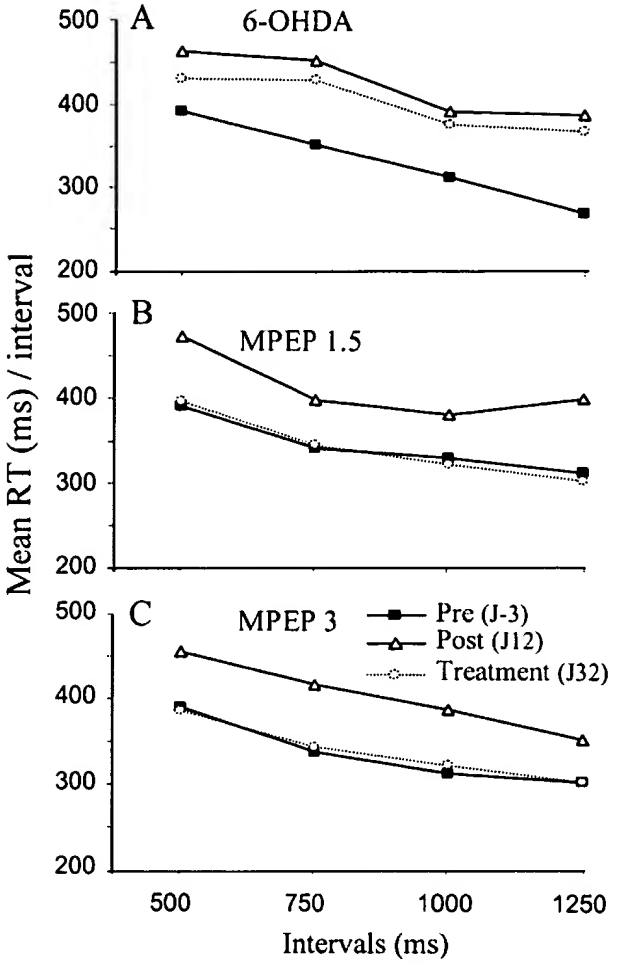

Figure 4. Effects of 6-OHDA lesion and MPEP (1.5 and 3 mg/kg) chronic treatment on reaction time. Mean RTs are plotted as a function of the various intervals preceding the stimulus onset for the three groups of animals [6-OHDA (n = 10), MPEP 1.5 mg/kg (n = 7), and MPEP 3 mg/kg (n = 10)]. Mean RTs were measured during three representative sessions: a preoperative day (*J-3*) and two postoperative days (*J12* and *J32*) corresponding to the lesion effect without treatment in comparison with the end of chronic treatment, respectively.

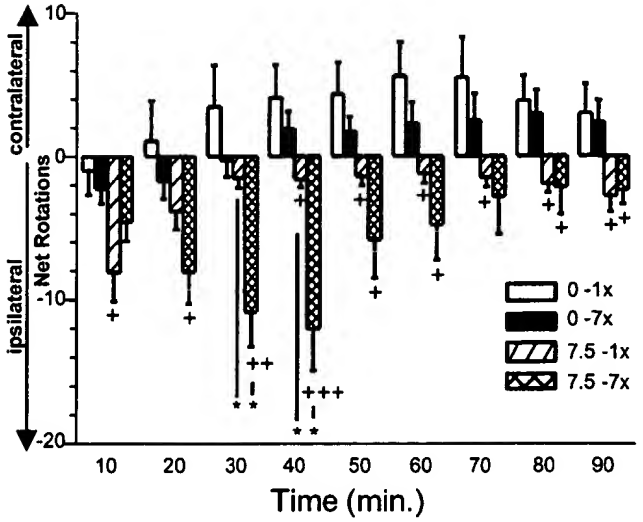

Figure 5. Acute or chronic treatment of MPEP on turning behavior. Error bars represent the mean (±SEM) number of net rotations (= the number of contralateral minus ipsilateral rotations; positive value indicates contralateral turning preference and a negative value indicates ipsilateral turning preference) per 10 min interval during 90 min of registration after a single (*1*×) or chronic (*7*×) application of vehicle (0.5% methylcellulose) or MPEP (7.5 mg/kg, p.o.). $^{+}p < 0.05$; $^{++}p < 0.01$; $^{+++}p < 0.01$ versus respective (*1x* or *7x*) vehicle treatment (unpaired t test); $^{**}p < 0.01$ for the indicated comparison (paired t test).

of PD and the dyskinetic movements induced by long-term L-DOPA treatment (Benabid et al., 1994; Limousin et al., 1995a,b). In parallel, pharmacotherapies have been dedicated to modulating glutamate transmission with compounds acting at the mGluR subtypes in addition to the classical DA replacement therapy.

Because of its strategically interesting expression pattern within the basal ganglia, the mGluR5 subtype was considered a serious target for the pharmacological treatment of PD. *In situ* hybridization and immunohistochemistry studies reported a high level of mGluR5 expression in the striatum and a moderate labeling in the STN and its output structures, the external segment of the globus pallidus, GPi, and SNr in the rat (Testa et al., 1994; Shigemoto and Mizuno, 2000). Furthermore, in these basal ganglia structures, the group I mGluRs are located primarily at postsynaptic sites (Tallaksen-Greene et al., 1998; Hanson and Smith, 1999; Awad et al., 2000; Hubert et al., 2001). MPEP could therefore produce its beneficial anti-parkinsonian effect by reducing the excessive glutamatergic drive on GABAergic output pathways of the basal ganglia and thereby counteracting the shutdown of thalamocortical projections. In addition, MPEP might also directly inactivate mGlu5 receptors in the striatum and modulate the neurotransmission of the output pathways. In agreement with this, recent behavioral studies have pointed out the role of group I or II mGluRs agonists in the nucleus accumbens in the generation and regulation of locomotion in a dopamine-dependent manner (Sacaan et al., 1992, Attarian and Amalric, 1997; Vezina and Kim, 1999; Swanson and Kalivas, 2000; Breysse et al., 2002). A possible mechanism to explain the restoration of normal motor function by MPEP is an increase in the DA level in the striatum, and this could be achieved in a partially inactivated DA system. This is further suggested in our partial model of 6-OHDA lesions in which intact DA nerve terminals may still be functionally active and possibly modulated by MPEP treatment. The present model involves a progressive neurodegeneration of DA neurons that may represent the preclinical stages of PD. These dopaminergic lesions are known to produce specific impairments in motor planning that were not compensated for several weeks after the lesion (Amalric and Koob, 1987; Amalric et al., 1995), and the major deficits, reflected by an increase in the number of delayed responses, are also observed in parkinsonian patients tested in similar RT tasks. Under these conditions, the behavioral progressive recovery of preoperative performance observed after chronic MPEP is similar to that obtained after chronic L-DOPA treatment in the same RT task and might constitute an alternative to the classic L-DOPA therapy (Maurin et al., 2001).

The beneficial effect of MPEP on parkinsonian-like deficits might also be explained by an interaction between group I mGlu and NMDA receptors that are known to be colocalized in different areas of the CNS such as the hippocampus, striatum, STN,

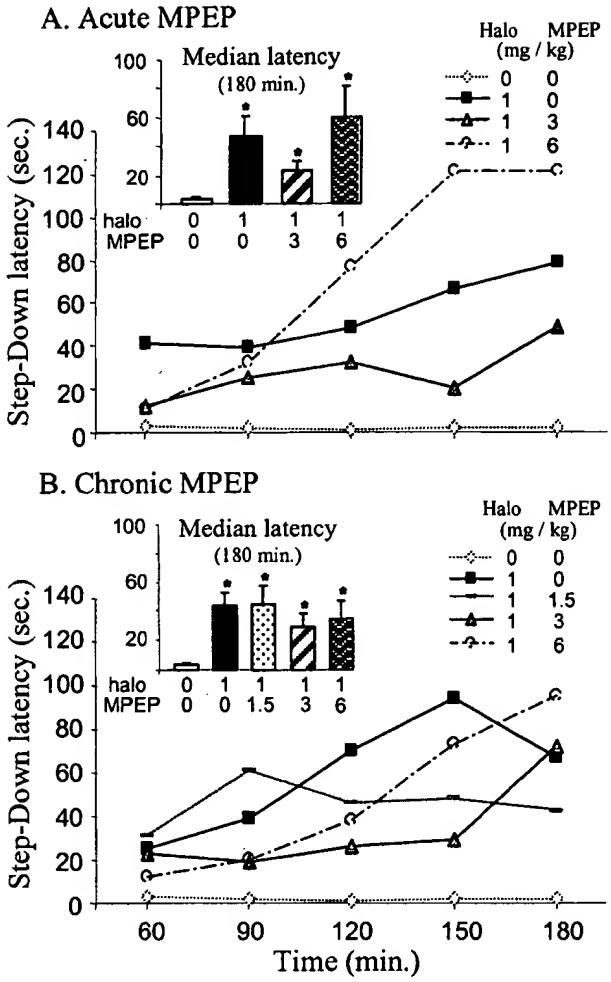

Figure 6. Acute or chronic MPEP treatment on haloperidol-induced catalepsy. The animals were tested every 20 min after the haloperidol injection (1 mg/kg, i.p.). *A*, Effects of acute treatment of MPEP on the median latency to step down a rod located 9 cm above the floor (n = 6 by dose of MPEP; n = 8 for control animals). *Inset* shows the mean median latency (±SEM) during the total duration of the test (180 min). *B*, Effects of chronic MPEP treatment at doses of 1.5, 3, and 6 mg/kg (n = 8 each dose) on catalepsy.

and thalamus in rat (Fitzjohn et al., 1996; Doherty et al., 1997; Pisani et al., 1997; Awad et al., 2000; Salt and Binns, 2000). For instance, Awad et al. (2000) reported that the activation of mGluR5 has direct excitatory effects and potentiates NMDA receptor currents in neurons of the STN, thereby increasing burst firing. Blockade of mGluR5 receptors with MPEP will thus exert a negative modulatory action on NMDA responses, thus reducing STN activity, and ultimately improve the motor deficits produced by the dopaminergic lesion.

Pharmacological attempts to reduce excessive glutamate activity in the basal ganglia have been achieved with NMDA receptor blockade in animal models of PD (Greenamyre et al., 1991; Schmidt et al.,1992; Baunez et al., 1994; Ossowska, 1994; Amalric et al., 1995; Ossowska et al., 1996; Lorenc-Koci et al., 1998). These studies have yielded only limited success, basically because of considerable side effects induced by some compounds that were envisioned to have therapeutic benefit (Schmidt et al., 1992; Schmidt, 1994; Amalric et al., 1995). Clinical data suggest that undesirable effects might also be predicted to occur in humans (Ossowska et al., 1994; Andine et al., 1999). The subtype selectivity of MPEP and the modulatory action of the mGluRs on glutamatergic transmission may be responsible for the beneficial effect displayed by MPEP in comparison with NMDA antagonists.

A 3 week chronic treatment of MPEP is necessary to reverse the deficits produced by the bilateral 6-OHDA model of PD in the RT task, whereas acute injection has no effect. Furthermore, chronic MPEP administration produced a clear ipsilateral rotational response, whereas only a weak effect was observed previously in the same unilateral 6-OHDA lesion model after an acute injection of MPEP (Spooren et al., 2000). The fact that mGluRs mediate modulatory effects of synaptic transmission by the activation of a number of intracellular metabolic pathways (Pin and Duvoisin, 1995; Schoepp et al., 1999) may explain these findings. Recent molecular studies indeed suggested an important role for Homer protein complexes in the regulation of trafficking and surface expression of group I mGluRs (Roche et al., 1999; Xiao et al., 2000). Behavioral correlates of this regulation have been suggested by Swanson et al. (2001) to explain the reduction of locomotor activation induced by a group I mGluR agonist in the nucleus accumbens after repeated cocaine administration. This chronic treatment produced long-term attenuation of group I mGluR function, and this diminished function was associated with decreased levels of mGluR5 and Homer 1b/c protein. In line with the idea that Homer 1a/b/c proteins are involved in the targeting of mGluR5 to the dendritic sites and axons and that this effect is regulated by neuronal activity (Ango et al., 2000), we suggest that MPEP chronic treatment acts in reducing neuronal hyperactivity in the different basal ganglia structures by dynamically regulating mGluR5 distribution in the neurons. This may lead to a stronger reduction of the transduction pathway than acute blockade with MPEP (Ango et al., 2001). This effect might not be observed in a pharmacological model of PD using DA receptor antagonists. Indeed, it was reported recently that acute MPEP treatment was able to antagonize the catalepsy and muscle rigidity induced by haloperidol (Ossowska et al., 2001). The present findings using a similar cataleptic test show that the reversal of catalepsy is clearly dependent on the dose tested and that chronic treatment with MPEP does not potentiate the antiparkinsonian-like effect of acute administration. These discrepant results might be explained by the different doses of haloperidol used (0.5 vs 1 mg/kg in the present study). As suggested above, a large blockade of DA receptors would prevent the functional interaction between group I mGluRs and DA activity existing in normal conditions (Meeker et al., 1998; David and Abraini, 2001).

In conclusion, the data presented here suggest that mGluR5 may be a particularly interesting target for modifying the glutamatergic hyperactivity within basal ganglia circuitry observed in PD. Chronic treatment with MPEP when used at an appropriate dosage may be considered a nonsurgical approach to the treatment of PD without adding side effects. Combined long-term treatment with subthreshold doses of MPEP and NMDA receptor antagonists and L-DOPA could therefore provide new therapeutic benefits that bypass the problems inherent with DA therapy.

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Reinforcing and locomotor stimulant effects of cocaine are absent in mGluR5 null mutant mice

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Both ionotropic and metabotropic glutamate receptors (mGluRs) are involved in the behavioral effects of pyschostimulants[1–3]; however, the specific contributions of individual mGluR subtypes remain unknown. Here we show that mice lacking the mGluR5 gene do not self-administer cocaine, and show no increased locomotor activity following cocaine treatment, despite showing cocaine-induced increases in nucleus accumbens (NAcc) dopamine (DA) levels similar to wild-type (WT) mice. These results demonstrate a significant contribution of mGlu5 receptors to the behavioral effects of cocaine, and suggest that they may be involved in cocaine addiction.

Both acute and repeated cocaine administration increase glutamate concentrations in the NAcc[4,5], a brain region associated with the reinforcing and locomotor effects of cocaine[6,7]. Systemic and brain injections of non-selective mGluR agonists and antagonists mediate baseline and psychostimulant-induced locomotor activity[1–3]. mGluR5 is highly expressed in the NAcc[8], and repeated systemic cocaine treatment increases mGluR5 mRNA levels in the NAcc shell and dorsolateral striatum[9]; however, the functional role of mGluR5 in cocaine effects remains unknown.

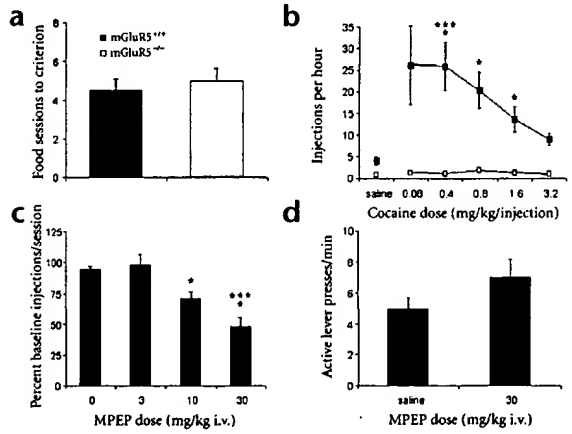

Fig. 2. Food and cocaine reinforcement. (**a**) Acquisition to criterion of food-reinforced lever pressing did not differ in mGluR5 WT (n = 5) and null mutant (n = 6) mice. Values represent mean ± s.e.m. (Student's t-test). (**b**) Cocaine SA in mice shown in (**a**). Values represent mean number of injections for 2 sessions at each dose ± s.e.m. $*p < 0.05$ versus saline within genotype; $***p < 0.05$ WT at 0.4 versus WT at 3.2 mg/kg/injection (Bonferroni-corrected Student's t-tests after two-way repeated-measures ANOVA). (**c**) MPEP dose-dependently decreased cocaine SA in C57Bl/6J mice (n = 5). Values represent mean percent of baseline number of injections per 1 h session at 0.8 mg/kg/injection, $*p < 0.05$ versus saline; $***p < 0.05$ versus 3 mg/kg MPEP (means comparisons after one-way ANOVA). (**d**) MPEP (30 mg/kg i.v.) had no effect on the number of food-reinforced lever presses per minute in C57Bl/6J mice (n = 5) (repeated-measures t-test). For detailed methods, see the supplementary information page of *Nature Neuroscience* online.

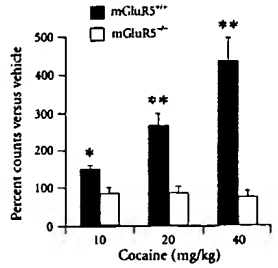

Fig. 1. Locomotor response to cocaine in mGluR5 WT (n = 14) and null mutant (n = 16) mice. Values represent mean percent activity counts ± s.e.m. $*p < 0.05$ versus saline; $**p < 0.01$ versus saline (Dunnett's test after two-way repeated-measures analysis of variance; ANOVA). For detailed methods, see the supplementary information page of *Nature Neuroscience* online.

To investigate this role, we first examined the locomotor responses to cocaine in F5 generation mGluR5 WT and null mutant mice. Baseline locomotor activity did not differ between mutant and WT mice (mutant, 2710 ± 642; WT, 2393 ± 479, mean horizontal activity counts/45-min session ± s.e.m.). Cocaine induced a significant, dose-dependent increase in locomotor activity in WT mice, but did not alter locomotor activity in mutant mice at any time point or dose tested (Fig. 1). Although mice received repeated cocaine exposure that may have induced some behavioral sensitization in WT mice, locomotor activity was not increased in mutant mice. Our results indicate that mGluR5 is essential for cocaine-induced hyperactivity.

To investigate whether the reinforcing properties of cocaine were affected by the mGluR5 mutation, we examined intravenous cocaine self-administration (SA) in WT and mutant mice. Acquisition of a discriminated two-lever food-reinforced task did not differ between WT and mutant mice (Fig. 2a). When intravenous cocaine was substituted for food, WT mice acquired stable cocaine SA across a typical dose range[10], but mutant mice did not self-administer cocaine at any dose tested (Fig. 2b). Active lever responding in mutant mice extinguished within three to five sessions at all cocaine doses, and no mutant mouse acquired stable SA, suggesting that the reinforcing properties of cocaine are absent in mice lacking mGluR5. Data from the food training suggest that the failure to acquire cocaine SA was not due to an inability to learn the lever-press task, and that the reinforcing properties of food are unchanged in mutant mice.

The selective mGluR5 antagonist 2-methyl-6-(phenylethynyl)-pyridine (MPEP)[11] dose-dependently decreased cocaine SA (Fig. 2c) in C57Bl/6J mice. The effect of MPEP was specific to cocaine reinforcement, as MPEP had no effect on the rate of food-reinforced lever pressing (Fig. 2d) under the same schedule of reinforcement as during cocaine SA. These results suggest that

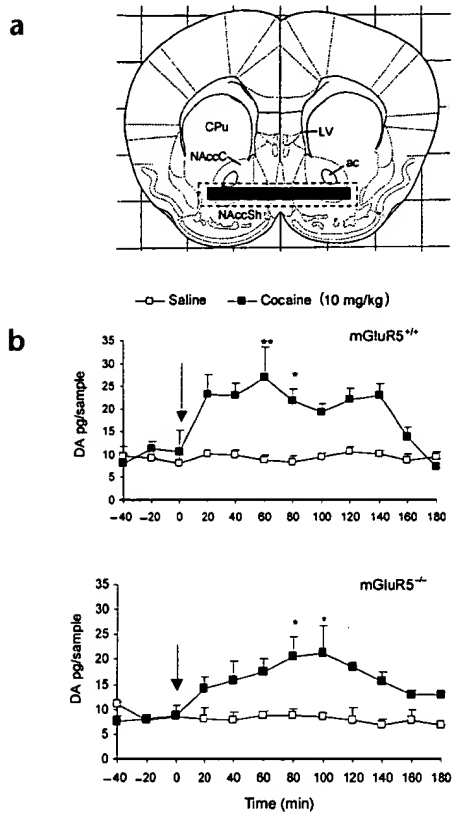

Fig. 3. Extracellular NAcc DA levels in mGluR5 WT and null mutant mice. **(a)** Dialysis probe location. Solid and dashed boxes indicate the minimum and maximum extent of probe placements. ac, anterior commissure; CPu, caudate putamen; LV, lateral ventricle; NAccC, nucleus accumbens core; NAccSh, nucleus accumbens shell. **(b)** DA level analysis. 10 µl samples were collected every 20 min from WT and mutant mice (saline, n = 3/genotype; cocaine, n = 3/genotype) and were analyzed by HPLC. Values represent mean pg/sample DA ± s.e.m. $*p < 0.05$; $**p < 0.01$ cocaine versus saline at the same time point within genotype (Bonferroni-corrected Student's t-test after separate two-way repeated-measures ANOVA). Extracellular DA levels were not significantly different between cocaine-treated WT and mutant groups (repeated-measures ANOVA, no significant main effects or interaction). For detailed methods, see the supplementary information page of *Nature Neuroscience* online.

the absence of cocaine SA in mutant mice was due to the loss of mGlu5 receptors and not to developmental alterations resulting from the genetic mutation.

Numerous studies have reported that self-administered cocaine increases DA levels in the ventral striatum[6], and psychostimulant-induced locomotor activity and increased mesoaccumbens DA levels in mice are closely correlated[12]. Because mutant mice showed neither a cocaine-induced locomotor response nor cocaine SA, we examined the effect of cocaine on NAcc DA levels using microdialysis in conscious, freely moving WT and mutant mice[12]. Wild-type and mutant mice had similar basal levels of extracellular DA. Cocaine-induced (10 mg/kg, i.p.) increases in extracellular DA levels did not differ between WT and mutant mice (Fig. 3b). These results suggest that the absence of mGluR5 affects neither baseline nor cocaine-induced increases in NAcc DA levels.

To ensure that changes in responses to cocaine in mutant mice were not due to mGluR5 mutation-induced alterations in dopaminergic elements, we investigated the brain distribution and expression of DA receptors and the DA transporter (DAT) in mutant and WT mice. No differences were found between WT and mutant mice in binding of the selective radiolabeled compounds ^{3}H-SCH23390 to D1-like and ^{3}H-YMO91512 to D2-like DA receptors and 3-WIN35,428 to the DAT, or in expression of D1 or D2 DA receptor mRNA examined by *in situ* hybridization (data not shown). These findings indicated that the expression and distribution of DA receptors and of the DAT are not altered in the mutant mice.

Several neurotransmitters and peptides contribute to cocaine dependence[6,7]. Although evidence supports a primary involvement for DA, the precise roles of specific DA receptor subtypes remain unclear. D1- or D2-like DA receptor antagonists reduce the reinforcing effects of cocaine[6,7]; however, D1 receptor mutant mice acquire a cocaine-conditioned place preference[13] and D2 receptor mutant mice self-administer cocaine (S.B. Caine *et al.*, *Soc. Neurosci. Abstr.* 26, 681.8, 2000). Likewise, the exact mechanisms of the mGluR5 contribution to cocaine dependence are not known. It is possible that glutamate acts in synergy with mesolimbic DA afferents into the NAcc to mediate the effects of cocaine. Acute cocaine increases extracellular NAcc dopamine[14] and glutamate[4] levels, and these effects are enhanced after repeated cocaine administration[5,14]. Excitatory amino acid (most likely glutamatergic) and mesolimbic dopaminergic terminals form synapses on single NAcc neurons[2,3]. Nucleus accumbens output neurons express both DA[2,6,7] and mGlu5[8] receptors. As has been suggested for locomotor activity[2], mGluR subtypes expressed by NAcc projection neurons may interact with dopaminergic inputs through their respective intracellular signaling pathways to influence the reinforcing effects of cocaine. Regardless of the specific mechanisms involved, the present results suggest that mGluR5 is essential in cocaine SA and locomotor effects.

Note: Supplementary methods are available on the Nature Neuroscience web site (http://neuroscience.nature.com/web_specials).

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Exacerbation of Neuronal Cell Death by Activation of Group I Metabotropic Glutamate Receptors: Role of NMDA Receptors and Arachidonic Acid Release

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Both ionotropic and metabotropic glutamate receptors have been implicated in the pathogenesis of neuronal injury. Activation of group I metabotropic glutamate receptors (mGluR) exacerbates neuronal cell death, whereas inhibition is neuroprotective. However, the mechanisms involved remain unknown. Activation of group I mGluR modulates multiple signal transduction pathways including stimulation of phosphoinositide hydrolysis, potentiation of NMDA receptor activity, and release of arachidonic acid. Here we demonstrate that whereas activation of group I mGluR by (*S*)-3,5-dihydroxyphenylglycine (DHPG) potentiates NMDA-induced currents and intracellular calcium increases in rat cortical neuronal cultures, partial effects of group I mGluR activation or inhibition on neuronal injury induced by oxygen–glucose deprivation remain despite NMDA receptor blockade. DHPG stimulation also increases basal arachidonic acid release from rat neuronal–glial cultures and potentiates injury-induced arachidonic acid release in these cultures. Thus, activation of group I mGluR may exacerbate neuronal injury through multiple mechanisms, which include positive modulation of NMDA receptors and enhanced release of arachidonic acid.

Key Words: **CNS injury; ischemia; trauma; calcium; neuronal–glial culture; signal transduction.**

INTRODUCTION

CNS injury is characterized by activation of both ionotropic and metabotropic glutamate receptors. The former are directly linked to cation channels, whereas the latter are coupled to G-proteins (48). Numerous reports have demonstrated the neuroprotective effects of ionotropic glutamate receptor antagonists following trauma or ischemia, both *in vitro* (35, 42) and *in vivo* (21, 22, 55). Modulation of metabotropic glutamate receptors (mGluR) also affects neuronal survival following injury, with the result depending upon the mGluR group involved and the type of injury model used (1, 9, 23, 26, 41, 47, 51).

A role for group I mGluR in neuronal injury has been established in a variety of *in vitro* and *in vivo* models. *In vitro,* activation of group I mGluR exacerbates, whereas inhibition of group I mGluR attenuates, neuronal injury induced by application of NMDA (8, 9), oxygen–glucose deprivation (OGD) (3, 9, 47), or mechanical trauma (41, 43). Similar results have been described *in vivo,* including models of ischemia–reperfusion (51) and traumatic brain injury (26, 41).

Much early work relating to signal transduction pathways activated by group I mGluR focused on phospholipase C, activation of which results in phosphoinositide (PI) hydrolysis, liberation of intracellular calcium stores, and stimulation of protein kinase C (13, 48). Yet it has been recognized that other pathways may also be induced by group I mGluR; these include activation of adenylyl cyclase, release of arachidonic acid, stimulation of phospholipase D, potentiation of NMDA receptors, and modulation of calcium and potassium channels (13, 48). However, many of these earlier studies were performed before the advent of subtype selective agonists and antagonists, utilizing transfection studies in cell lines to isolate each receptor subtype. Thus, the role of such signal transduction pathways in mediating effects of group I mGluR in neurons or during neuronal injury remains largely unexplored.

We have previously demonstrated that activation of group I mGluR increased phosphoinositides in neuronal–glial cultures and that antisense oligonucleotides directed against both mGluR1 and mGluR5 attenuated this response (41). Interestingly, antisense directed against mGluR1, but not mGluR5, selectively attenu-

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ated neuronal injury following mechanical trauma *in vitro* (41), despite similar functional effects of each oligonucleotide on PI hydrolysis. This finding suggests that activation of phospholipase C may not be a primary mechanism underlying the modulation of neuronal injury by group I mGluR. In the present studies, two possible mechanisms by which group I mGluR may exacerbate neuronal injury were evaluated. Using rat cortical neuronal and neuronal-glial cultures, we examined the effects of group I mGluR activation on NMDA receptor-mediated responses and arachidonic acid release.

MATERIALS AND METHODS

Neuronal-Glial Cultures

Glia were prepared from 1- to 3-day-old Sprague–Dawley rat cortices (Taconic Farms) and neurons were prepared from 18-day-old Sprague–Dawley rat embryonic cortices as previously described in detail (44). Briefly, cortices dissociated from 1- to 3-day-old rats were seeded in 96-well Primaria microplates (Falcon) and resulting glia were allowed to grow to confluency. Cortices were dissociated from 18-days-old rat embryos and individual cells ($2–2.5 \times 10^6$ cells/ml) were plated on a layer of confluent glial cells; they were then dissociated in Hanks' balanced salt solution without calcium or magnesium (Mediatech) supplemented with 10 mM Hepes (pH 7.0; Biofluids) and 1 mM sodium pyruvate (Biofluids). Cultures were fed twice per week by replacement of one-third of medium with minimal essential medium with Earle's salts (Mediatech) supplemented with 10% equine serum (HyClone Laboratories), 27.5 mM Hepes (pH 7.2), 2 mM glutamine (Biofluids), 20 mM glucose (Biofluids), and 1% antibiotic–antimycotic solution (Biofluids). Cytosine-β-D-arabinofuranoside (10 μM; Sigma) was added during the first feeding to stop further glial proliferation. After 10 days *in vitro* (DIV), glutamine concentration was reduced to 1 mM and equine serum was omitted. Cultures were incubated at 37°C in humid atmosphere with 4% CO_2. Neuronal–glial cultures were used at 19–21 DIV.

Cortical Neuronal Cultures

Neocortices from 18-day-old Sprague–Dawley rat embryos (Taconic Farms) were used to prepare neuronal cultures. Individual neurons were obtained as detailed above. After dissociation, cell suspension was diluted with Neurobasal medium (NBM; Life Technologies) supplemented with 25 μM glutamate (Sigma), 0.5 mM glutamine, 1% antibiotic–antimycotic, and 2% B27 supplement (Life Technologies). Cells were seeded at 1×10^6 cells per milliliter onto microplates (96-well; Corning) or circular coverslips (12 or 25 mm; Fisher Scientific) precoated with 10 μg/ml poly-D-lysine (Sigma). Pure neuronal cultures were fed on day 4 *in vitro* by the addition of an equal volume of NBM supplemented with 0.5 mM glutamine, 1% antibiotic–antimycotic, and 2% B27 supplement. Medium volume was then adjusted to 100 μl. Mature neuronal cultures were used at 11–14 DIV.

Qualitative Reverse-Transcription Polymerase Chain Reaction (RT-PCR)

The expression profiles for mGluR1, mGluR5, and NMDA NR1 mRNA were analyzed in neuronal (11 DIV) and neuronal–glial (18 DIV) cultures in 96-well microplates by qualitative RT-PCR. Cells were lysed using TRIzol reagent (Life Technologies) and chloroform (Sigma) was added to lysates. Samples were centrifuged at 13,000*g* at 4°C for 15 min and an equal volume of isopropanol (Sigma) was added to the aqueous phase. After incubation at −20°C, RNA was subjected to ethanol (Sigma) precipitation and RNA concentration was determined spectrophotometrically for each sample.

Complementary DNA (cDNA) was generated for each sample. Twenty micrograms of RNA was ethanol precipitated and resuspended in deionized water. DNA present in the sample was degraded by incubation with 0.2 U/μg RNA of RNase-free DNase I (Promega) in cDNA synthesis buffer (Life Technologies) at 37°C for 1 h. cDNA was produced by adding the following to each sample: 5 mM DTT (Life Technologies), 500 μM dNTPs (Sigma), 4 μM oligo(dT) primer (15-mer; Life Technologies), 4 μM random primer (10-mer; Life Technologies), and 2 μl Moloney murine leukemia virus reverse transcriptase (Life Technologies). Reactions were incubated at 37°C for 2 h, and reverse transcriptase was inactivated by incubating samples at 70°C for 10 min.

PCR was performed using 1/10 of the cDNA volume and 30 pmol of the following specific primer pairs: for mGluR1, 5′-CCCCTGTTCCTGGCTGATTC-3′ and 5′-AAAGGAGAAGGAGGCGTCAG-3′; for mGluR5, 5′-GCTTCACAGCCAACATCTCC-3′ and 5′-TTTGGGAGAGGATGGGATGC-3′; and for NMDA NR1, 5′-GGCCGTGCTGGAGTTTGAGG-3′ and 5′-CCCCGGTGCTCGTGTCTTTG-3′. The following program was used: initial denaturing at 95°C for 2 min, subsequent denaturing at 94°C for 2 s, annealing at 55°C for 15 s, primer extension at 72°C for 45 s, and final primer extension at 72°C for 2 min. One-third of the reaction volume was loaded onto a 1.5% agarose gel containing 0.5 μg/ml ethidium bromide. Electrophoresis was performed at 5 mV/cm. DNA was visualized by ultraviolet transillumination at 300 nm. Images were captured using Speedlight gel documentation system (Hoefer).

To confirm the sequence of RT-PCR results, each product was cloned into PCR 2.1 plasmid using a TA

cloning kit (Invitrogen). Resulting plasmids were transfected into competent NM522 *Escherichia coli*. Positive clones were picked and plasmid DNA was isolated using Wizard *Plus* miniprep DNA purification system (Promega) and sequenced using ABI Prism dye terminator cycle sequencing ready reaction kit (Perkin Elmer) following manufacturer's guidelines.

Electrophysiology

Neuronal cultures grown on 12-mm coverslips were used for electrophysiological studies. Borosilicate glass recording pipettes were pulled to a resistance of 6–8 MΩ. Coverslips containing neurons were transferred to a stage of an inverted microscope and placed into a perfusion bath of extracellular recording solution (ERS) containing 145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 10 μM $MgCl_2$, 0.02 mM glycine, 5 mM glucose, and 5 mM Hepes at pH 7.2 with NaOH. Osmolarity of ERS was adjusted to 325 mOsm with sucrose. The culture dish in the recording chamber was continuously perfused at a rate of 5 ml/min. Phase-contrast bright neurons were voltage-clamped at −60 mV in the whole-cell configuration using the patch-clamp technique (28) with a recording pipette containing 145 mM K–gluconate, 5 mM EGTA, 5 mM $MgCl_2$, 5 mM NaATP, 0.2 mM NaGTP, and 10 mM Hepes at pH 7.2 with KOH. NMDA and DHPG were applied directly through a gravity-fed Y-tubing delivery system placed within 100 μm of the recorded cell (45). Currents were monitored with a patch amplifier (EPC-7; List Electronics), filtered at 1.5 kHz (eight-pole low-pass Bessel; Frequency Devices), and digitized with the use of an IBM-PC computer with pCLAMP 6 software.

Calcium Imaging

NMDA-induced increases in intracellular calcium levels were monitored using an established method (31). Neuronal cultures grown on 25-mm coverslips were used for calcium imaging. Cultures were washed with ERS. Neurons were loaded with a calcium-sensitive fluorescent probe by incubating cells with 1 μM fura-2 AM (Molecular Probes) at 37°C for 1 h in 1 ml ERS supplemented with 1 mM $MgCl_2$ (ERSM). After loading, cultures were washed three times with ERSM. Coverslips containing neurons were then transferred to an inverted Zeiss microscope and baseline measurements were obtained in ERSM. Addition of an equal volume of ERSM had no effect on basal levels of intracellular calcium (data not shown). DHPG or vehicle was diluted in ERSM and added to cultures. After 2 min, NMDA diluted in ERSM was added to cultures. Fluorescent images were acquired at 334 and 380 nm at a rate of 1 ratio image per second using a Zeiss-Attofluor ratio arc imaging system (Zeiss) and a 40× Zeiss Fluor oil immersion objective lens.

Induction of in Vitro Mechanical Trauma

The induction of injury and the cellular response to this trauma model has been previously described in detail (2, 44). This model has been modified as outlined below. All drugs were added 30 min prior to injury. Media from neuronal–glial plates was replaced with a balanced salt solution (BSS) containing 116 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 1.0 mM NaH_2PO_4, 26.2 mM $NaHCO_3$, 0.01 mM glycine, and 10 mg/L phenol red. Control uninjured and trauma cultures were supplemented with 5.5 mM glucose. Ten millimolar 3-nitropropionic acid (3NP) was added to cultures used for trauma with 3NP and glucose deprivation (trauma + 3NP/GD) injury. Injury was induced by a specially designed punch device that produces 28 parallel cuts 1.2 mm in length at 0.5 mm intervals. Immediately following injury, cultures were returned to 37°C and 5% CO_2 and incubated for 60 min. Cultures were then washed seven times with BSS and the medium volume was adjusted to 100 μl by the addition of BSS containing 1% antibiotic–antimycotic. Glucose was added to a final concentration of 5.5 mM and cultures were incubated at 37°C for 24 h. Control uninjured sister cultures were treated identically with the exception of trauma and were used to estimate basal cell death.

Induction of Oxygen-Glucose Deprivation Injury

OGD injury was performed following an established protocol with minimal modifications (3, 25). In brief, atmosphere was evacuated from anaerobic system Model 1025 (Forma Scientific) and replaced with 5% CO_2, 10% H_2, and 85% N_2. Deoxygenated BSS was prepared by bubbling with the anaerobic gas mixture prior to starting experiments. Neuronal–glial cultures in 96-well microplates were transferred into the anaerobic chamber and washed with deoxygenated BSS. All drugs were diluted with deoxygenated BSS in the chamber. Control cultures were treated with vehicle diluted in deoxygenated BSS. After adding compounds to appropriate wells, cultures were placed in an incubator at 37°C within the chamber. Cultures were removed from incubator and washed with deoxygenated BSS and then removed from the chamber. "Reperfusion" was performed by immediately adding an equal volume of oxygenated BSS supplemented with 5.5 mM glucose, 1% antibiotic–antimycotic and vehicle or drug where appropriate. Cultures were then placed at 37°C for 24 h before cell death assessment.

Cell Death Assessment

Total cell death was estimated using lactate dehydrogenase (LDH) released into the culture medium as a biochemical marker. LDH release in these models correlates well with other markers of cell death including

trypan blue counts and increases in ethidium homodimer fluorescence (42, 44). LDH activity measurements were performed following an established protocol (41). Twenty-four hours after injury, medium was transferred to a 96-well microplate and diluted with LDH assay reagent containing 5 mM β-NAD (Sigma), 25 mM lactic acid (Sigma), 0.03% bovine serum albumin (Sigma), 100 mM Trizma (Sigma), and 0.9% NaCl, pH 8.45. Spectrophotometric analysis was performed at room temperature using a Ceres 900 microplate reader (Biotek Instruments, Inc.) measuring optical density at 340 nm over 250 s at 5-s intervals (50 readings per sample). Linear regression analysis provided an estimate of LDH activity. Basal or control LDH activity levels were subtracted from treatments prior to analysis.

Measurement of [^{3}H]Arachidonic Acid Release

Release of [^{3}H]arachidonic acid ([^{3}H]AA) was quantified following an established protocol (60) with minor modifications. Neuronal–glial cultures were incubated with 0.1 μCi/ml of [^{3}H]AA (Amersham; sp act 216 Ci/mmol) at 37°C for 24 h and then washed seven times with BSS. Cultures were incubated for 10 min with fatty acid-free bovine serum albumin (BSA) at a final concentration of 5 μg/ml to trap released [^{3}H]AA. Some experiments were performed in the presence of 50 μM thimerosal to prevent reacylation of released [^{3}H]AA. Cultures were then exposed to drug or vehicle or subjected to trauma + 3NP/GD as described below. Medium was collected at various time points and centrifuged at 12,000*g* for 5 min to remove cellular debris. Supernatants were transferred to scintillation tubes and released radioactivity was measured by liquid scintillation counting.

Drugs

(*R,S*)-1-Aminoindan-1,5-dicarboxylic acid (AIDA), (S)-4-carboxyphenylglycine (4CPG), dizocilpine (MK801), and (*S*)-3,5-dihydroxyphenylglycine (DHPG) were all purchased from Tocris Cookson. Tetrodotoxin (TTX) was purchased from Calbiochem.

RESULTS

Cortical Neurons Express both NMDA Receptors and Group I mGluR

We have previously demonstrated that neuronal–glial cultures express both NMDA NR1 and group I mGluR using RT-PCR and Western blot techniques (41, 44). The presence of NMDA receptors was confirmed in rat cortical neuronal and neuronal–glial cultures by qualitative RT-PCR using primers specific for NR1 (Fig. 1). NR1 expression is required for the assembly of functional NMDA receptors (39). Qualitative RT-PCR was also used to demonstrate the expression of mGluR1 and mGluR5 in both of these cultures (Fig. 1). The presence of NMDA receptors and group I mGluR were confirmed in cortical neuronal cultures by immunohistochemistry using antibodies specific for NMDA NR1, mGluR1, and mGluR5 (data not shown).

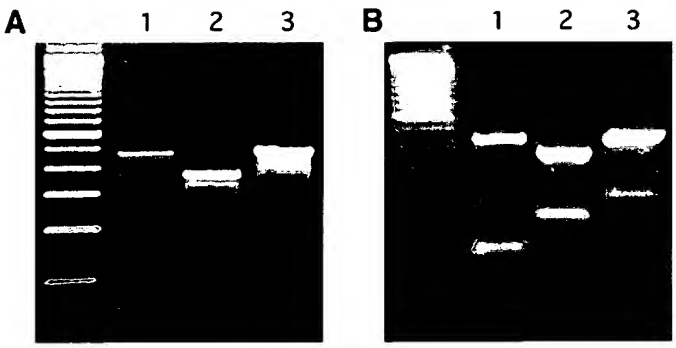

FIG. 1. Qualitative RT-PCR analysis of mRNA extracted from 18 DIV neuronal–glial (A) and 11 DIV neuronal (B) cultures using primers specific for group I mGluR (mGluR1 and mGluR5) or NMDA receptors (NR1). Both neuronal–glial (A) and neuronal cultures (B) express mGluR1 (lane 1), mGluR5 (lane 2), and NR1 (lane 3) mRNA. The specificity of all bands was confirmed by subcloning and sequencing as detailed under Materials and Methods.

Group I mGluR-Mediated Potentiation of NMDA Receptor Function

Cortical neurons grown on glass coverslips were transferred to a perfusion bath of BSS containing 0.5 μM TTX to inhibit synaptic currents. NMDA applied via Y-tubing to neurons in whole-cell configuration induced a large inward current that exhibited rapid desensitization (Fig. 2A). Cells that did not exhibit this response to NMDA receptor stimulation were not considered for further study. Selective activation of group I mGluR by DHPG via Y-tubing for 2 min did not generate any detectable current (Fig. 2A). Reapplication of NMDA after incubation with DHPG for 2 min induced a larger current compared with the initial NMDA response (Fig. 2A). The currents induced by NMDA after DHPG application exhibited no gross differences in kinetics or desensitization from NMDA currents generated before DHPG (Fig. 2A). Quantitation revealed a significant potentiation of maximal NMDA response after DHPG compared with currents induced by NMDA alone (Fig. 2B). NMDA receptor-mediated steady-state currents did not exhibit statistically significant enhancement by application of NMDA following incubation with DHPG (Fig. 2C).

Cortical neurons were loaded with the calcium-sensitive dye fura-2 AM. NMDA application in the presence of 0.5 μM TTX significantly increased intracellular calcium levels in these cells (Fig. 3A). Application of DHPG for 2 min did not appreciably alter intracellular calcium levels (Fig. 3A). Similar to the potentiation of NMDA receptor-mediated currents by activation of group I mGluR,

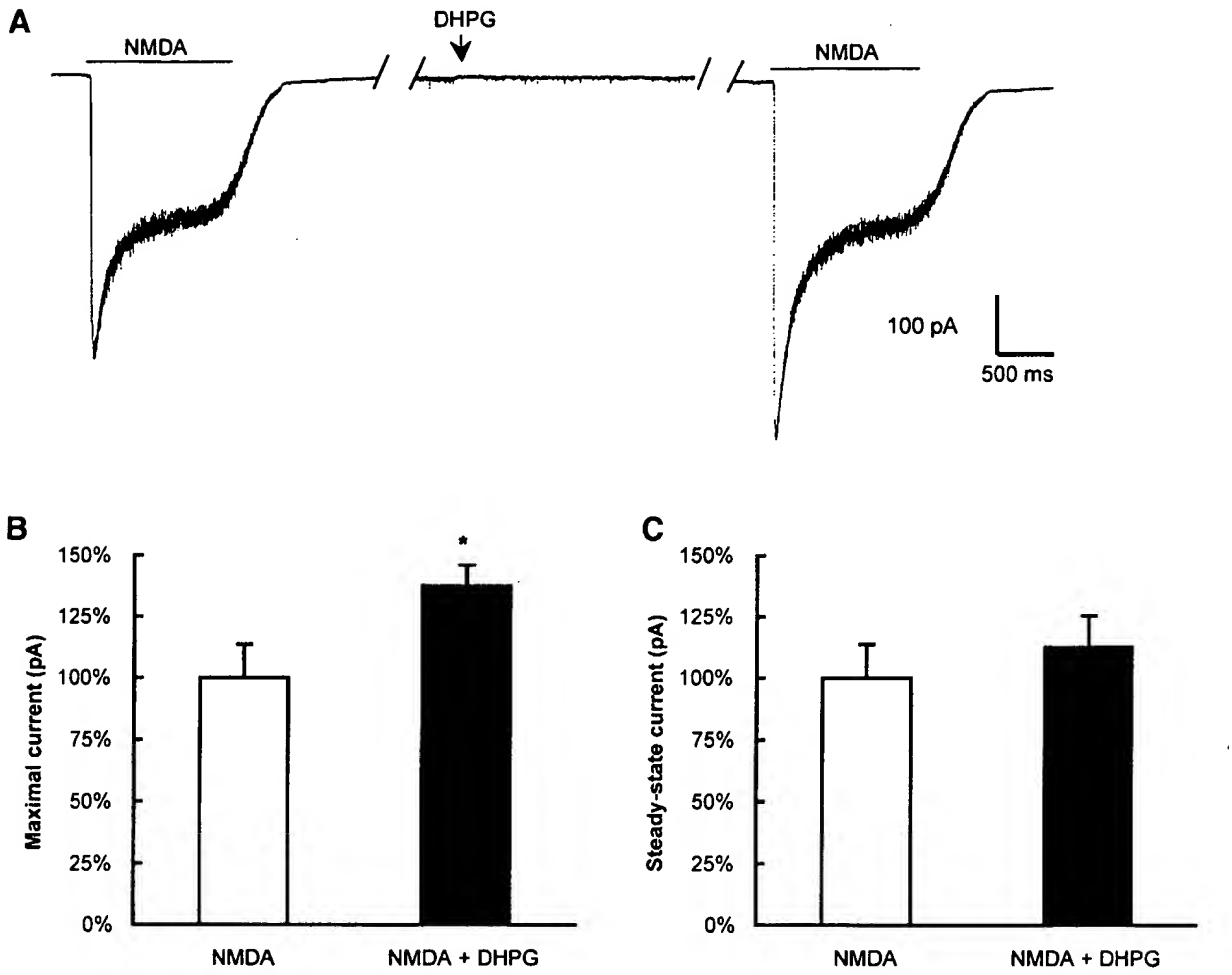

FIG. 2. Group I mGluR activation potentiates NMDA-mediated currents in 11 DIV neuronal cultures. (A) Representative current trace recorded from a single neuron voltage-clamped at a holding potential of −60 mV. After recording a baseline, NMDA (200 μM) was applied via Y-tubing. DHPG (200 μM) applied for 2 min did not generate any current (partial tracing shown). Reapplication of NMDA (200 μM) immediately after discontinuing DHPG application induced a larger current. (B) Maximal NMDA-induced current was significantly potentiated following administration of 200 μM DHPG for 2 min (filled bar) compared with NMDA alone (open bar). (C) Control NMDA-mediated steady-state current was unaffected by DHPG application. To block synaptic activity, 0.5 μM TTX was added to the extracellular recording solution during all experiments. All recordings were performed in the presence of 10 μM Mg^{2+}. Bars, mean + SEM, $n = 6$. Data are expressed as a percentage of NMDA-induced current before application of DHPG. $^*P < 0.05$, Student's t test.

DHPG significantly potentiated NMDA-induced increases in intracellular calcium (Fig. 3B).

Effects of Group I mGluR Modulation in the Presence of MK801 during Neuronal Injury

To determine whether group I mGluR-mediated potentiation of NMDA receptor function is important in the setting of neuronal injury, neuronal–glial cultures were subjected to OGD in the presence of NMDA receptor blockade. We have previously generated a dose–response curve for MK801 in the setting of mechanical trauma in these neuronal–glial cultures and found an EC50 of 10–50 nM (43, 44). Administration of MK801 completely inhibits NMDA toxicity (43) and blocks OGD toxicity (3, 25). We have also demonstrated that group I mGluR activation exacerbates OGD injury whereas group I mGluR inhibition protects against this injury (3). Blockade of group I mGluR by either AIDA or 4CPG provided significant protection against OGD-induced LDH release in the presence of a maximally effective dose of MK801 (Fig. 4A). In addition, activation of group I mGluR by DHPG significantly exacerbated OGD-induced injury in the presence of MK801 (Fig. 4B).

Effects of Group I mGluR Modulation on Trauma + 3NP/GD-Induced Injury

We have recently developed a novel injury model that combines mechanical trauma with "ischemic" conditions, which is characterized by both necrotic and apoptotic neuronal cell death (2). We examined whether modulation of group I mGluR had similar

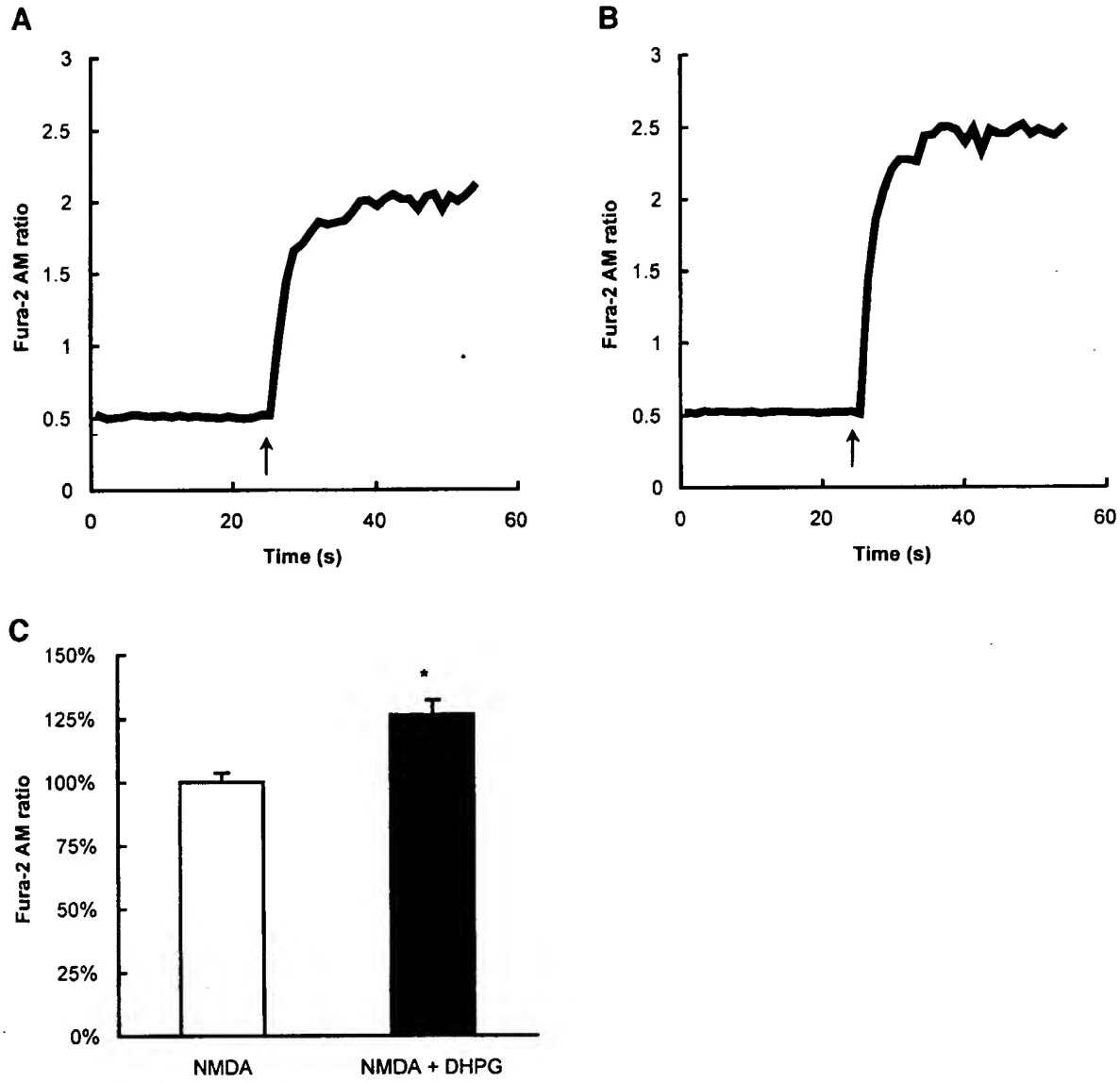

FIG. 3. Group I mGluR activation potentiates NMDA-induced increases in intracellular calcium in 11 DIV neurons. (A) Representative trace of average change in fura-2 AM ratio values after addition of 200 μM NMDA (arrow). (B) Preincubation with 200 μM DHPG for 2 min did not appreciably alter baseline intracellular calcium levels (partial tracing shown), but potentiated control NMDA-mediated rise in intracellular calcium. Values represent mean fura-2 AM ratio for 15–17 cells. (C) Quantitation demonstrates that DHPG (200 μM) significantly potentiated NMDA-induced fura-2 AM ratio values. Bars, means + SEM, n = 5–6 cultures (15–25 cells analyzed per culture). Data are expressed as a percentage of NMDA response. $^*P = 0.012$ vs control (two-tailed Mann–Whitney U test). TTX (0.5 μM) and 1 mM Mg^{2+} were included in the extracellular solution during all experiments.

effects in this injury model compared to either OGD or mechanical trauma. Inhibition of group I mGluR by AIDA provided significant protection against trauma + 3NP/GD, as indicated by LDH release at 24 h (Fig. 5A). Stimulation of group I mGluR by DHPG significantly exacerbated this injury at 24 h (Fig. 5B).

Potentiation of Basal- and Trauma + 3NP/GD-Induced Release of Arachidonic Acid by Group I mGluR Activation

Neuronal–glial cultures were preloaded with [^{3}H]AA as described above. Application of DHPG induced significant liberation of [^{3}H]AA into the culture medium compared with basal release (Fig. 6A). [^{3}H]AA levels began to return to basal levels after 30 min of stimulation with DHPG, which was the latest time point examined (Fig. 6A).

Release of [^{3}H]AA into medium of neuronal–glial cultures was significantly increased immediately following trauma + 3NP/GD injury (Fig. 6B). Increased levels of [^{3}H]AA were still detectable at 50 min (Fig. 6B). Application of DHPG during trauma + 3NP/GD significantly potentiated [^{3}H]AA release at both 30 and 60 min postinjury (Fig. 7). Similarly, OGD injury sig-

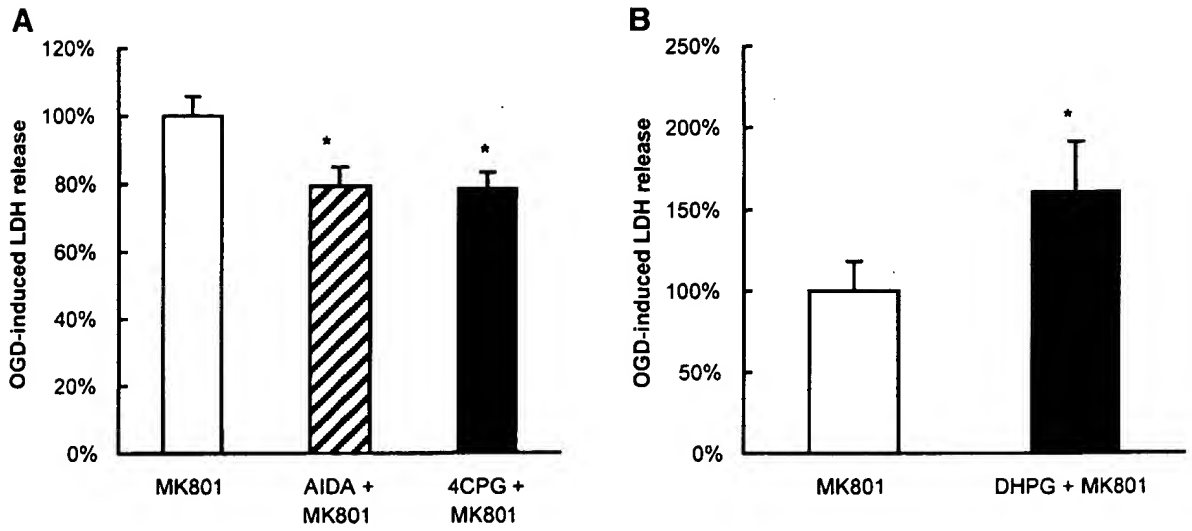

FIG. 4. Effects of group I mGluR modulation of OGD injury persist in the presence of NMDA receptor blockade. (A) Inhibition of group I mGluR by AIDA (200 μM; hatched bar) or 4CPG (30 μM; filled bar) in the presence of 10 μM MK801 induced significant protection over that produced by MK801 alone (open bar) 24 h after OGD. $^*P < 0.05$ vs control (ANOVA followed by Student–Newman–Keuls test). (B) Group I mGluR activation by DHPG (20 μM; filled bar) in the presence of MK801 significantly exacerbated OGD-induced LDH release at 24 h compared with MK801 alone (open bar). $^*P < 0.05$ vs control (Student's t test). Bars, means + SEM after basal LDH release subtraction, $n = 22$–31. Data are expressed as a percentage of OGD with MK801 treatment.

nificantly increased [^{3}H]AA at 60 min postinjury (control 100 ± 4 versus OGD 187 ± 35; $n = 6$–10; $P < 0.05$, Student's t test), and application of DHPG (20 μM) significantly potentiated this release at 60 min postinjury (OGD 100 ± 18 versus OGD + DHPG 149 ± 13; $n = 6$–10; $P < 0.05$, Student's t test).

DISCUSSION

The present studies demonstrate that the effects of group I mGluR stimulation include both potentiation of NMDA-induced responses and increases in arachidonic acid release from rat cortical cultures. In addition, we report that the exacerbating and neuroprotective effects of group I mGluR modulation following OGD injury partially persist in the presence of NMDA receptor blockade. These effects of group I mGluR modulation are consistent across injury models as similar effects were also observed following trauma + 3NP/GD injury. Taken together, our data suggest that multiple second messenger systems may be involved in group I mGluR modulation of neuronal injury.

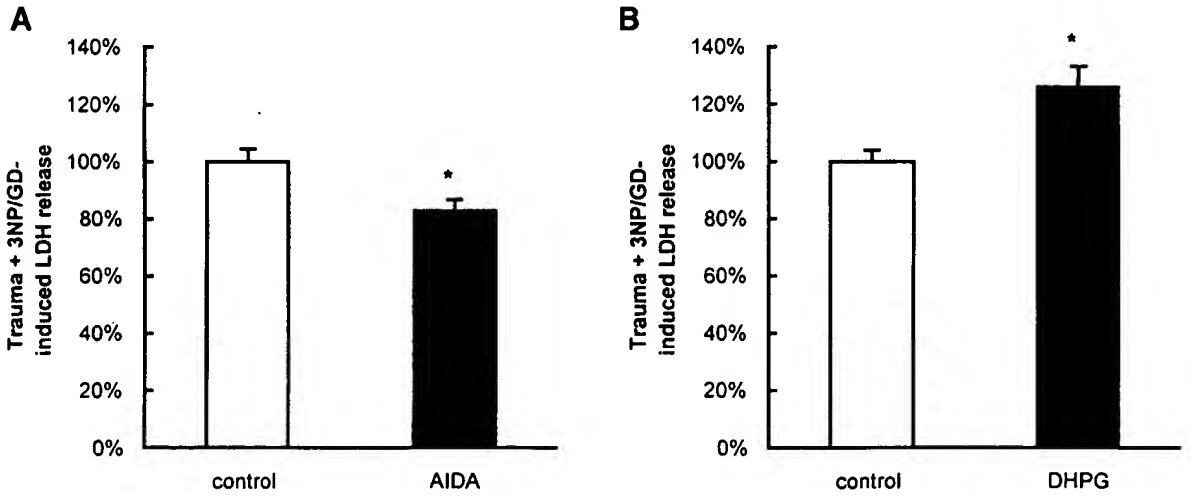

FIG. 5. Effects of group I mGluR modulation on trauma + 3NP/GD injury. (A) Group I mGluR inhibition by AIDA (200 μM) significantly decreased trauma + 3NP/GD-induced LDH release 24 h after injury. (B) Application of DHPG (20 μM) significantly worsened this injury. These effects are similar to those observed after group I mGluR modulation of OGD injury. Bars, means + SEM, $n = 19$–21. Data are expressed as a percentage of trauma + 3NP/GD (control). $^*P < 0.005$ vs trauma + 3NP/GD (Student's t test).

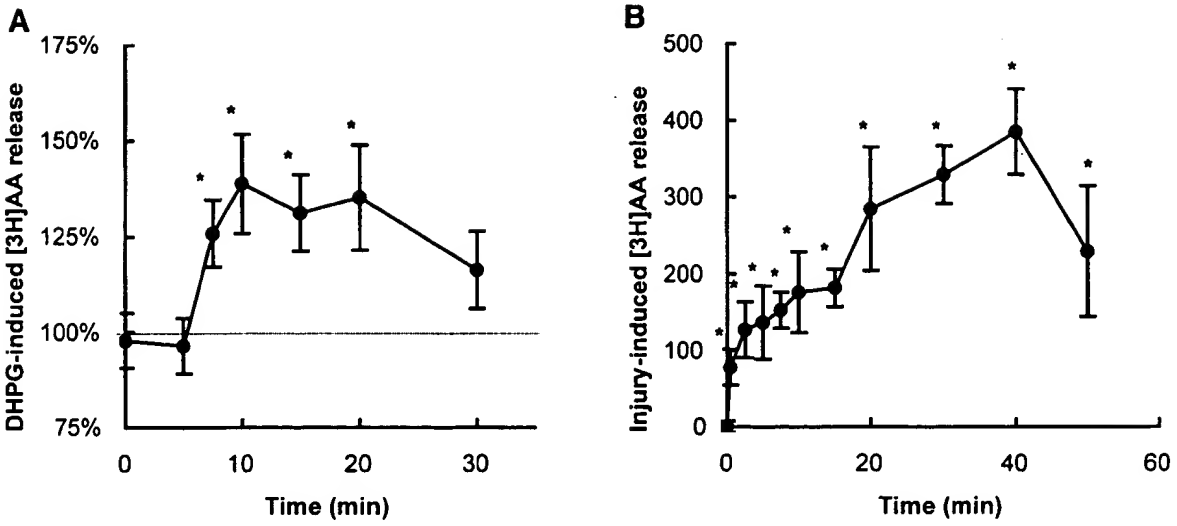

FIG. 6. Group I mGluR activation and trauma + 3NP/GD increase [^{3}H]arachidonic acid ([^{3}H]AA) release from neuronal–glial cultures. (A) Application of DHPG (20 μM) to 18 DIV neuronal–glial cultures significantly increases [^{3}H]AA detected in culture medium by 7.5 min. The *dashed line* indicates the level of basal release from control sister cultures. Values represent means ± SEM, $n = 8$. Data are expressed as a percentage of basal release. $^*P < 0.05$ vs basal levels (Student's t test). Experiments were performed in the presence of thimerosal to prevent reacylation of released [^{3}H]AA. (B) Trauma + 3NP/GD induces [^{3}H]AA release from neuronal–glial cultures. [^{3}H]AA levels are significantly increased in culture media immediately following trauma + 3NP/GD induction and remain significantly elevated at all time points examined. Values represent [^{3}H]AA detected in culture medium minus basal release in sister control cultures, $n = 3$. $^*P < 0.05$ vs basal release (Student's t test).

Early studies using *trans*-(±)-1-amino-1,3-cyclopentanedicarboxylic acid (ACPD), a relatively nonselective agonist, demonstrated that activation of mGluR potentiated NMDA-mediated currents in brain slices or cultured neurons from the hippocampus (4, 29), spinal cord (6, 57), striatum (49), or cortex (50). In contrast, two studies from independent laboratories have reported that ACPD attenuates NMDA receptor-mediated currents (12, 62). More recently, potentiation of NMDA-induced currents was attributed to activation of group I mGluR because the selective agonists DHPG and CHPG mimicked this effect when applied to brain slices or cultures from the hippocampus (24), spinal cord (32, 57, 58), striatum (49), or cortex (17).

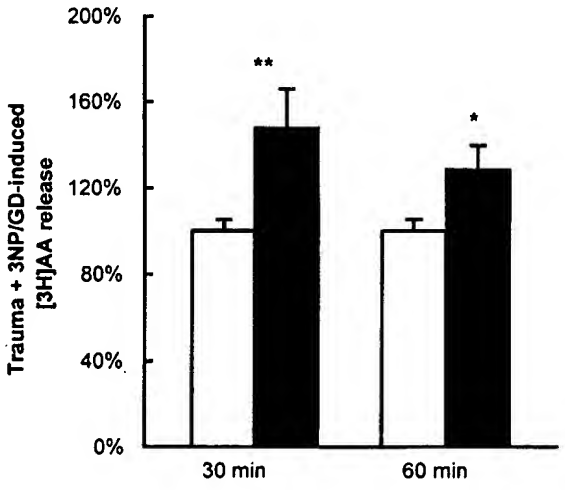

FIG. 7. Group I mGluR activation potentiates trauma + 3NP/GD-induced [^{3}H]arachidonic acid ([^{3}H]AA) release from neuronal–glial cultures. Treatment with 20 μM DHPG (filled bars) induced significant increases in [^{3}H]AA released into the culture medium at 30 and 60 min compared with trauma + 3NP/GD alone (open bars). Values represent means + SEM after subtraction of basal values, $n = 18$–21. Data are expressed as a percentage of trauma + 3NP/GD. $^*P < 0.05$ vs trauma + 3NP/GD; $^{**}P < 0.01$ vs trauma + 3NP/GD (Student's t test).

We were particularly interested in whether activation of group I mGluR would modulate NMDA receptors in rat cortical neurons, as this species has been used in many studies by us and others investigating the role of group I mGluR in neuronal injury. Application of DHPG significantly potentiated the maximal response induced by NMDA in whole-cell recordings of these neurons. This effect was not due to increased synaptic activity as a result of increased glutamate release, as all experiments were performed in the presence of TTX. Consistent with other studies, application of DHPG in the absence of NMDA did not generate any detectable current (62). Potentiation of NMDA-induced currents by DHPG did not appreciably alter the kinetics of NMDA receptor-mediated currents and this potentiation persisted up to 5 min after DHPG application, the latest time point examined. This is consistent with the report that enhancement of NMDA-generated currents by mGluR in rat spinal dorsal horn neurons persists up to 75 min (10).

Potentiation of NMDA receptor-mediated currents in rat cortical neurons was confirmed by calcium-imaging studies using fura-2 AM. No change in intracellular

calcium levels was detected after DHPG administration, indicating that group I mGluR activation alone does not lead to detectable increases in intracellular calcium in these neurons. However, increases in NMDA-induced intracellular calcium levels were observed after preincubation with DHPG. Again, all experiments were performed in the presence of TTX; thus, this increase was not a result of increases in synaptic transmission. As group I mGluR may enhance calcium channel activation in other cell types, such as cerebellar granule cells (11), a similar effect in cortical neurons cannot be excluded. Nonetheless, this appears unlikely given a lack of rise in intracellular calcium levels following the addition of DHPG alone, as well as the many reports indicating that the primary effect of group I mGluR activation on calcium channels is inhibition (37, 53, 54, 56).

In contrast to the data presented here, Yu *et al.* (62) reported a reversible attenuation of NMDA currents by selective group I mGluR agonists in cultured *mouse* cortical neurons. They also noted reduction of NMDA-induced increases in intracellular calcium in these cells by the addition of (*S*)-3-hydroxyphenylglycine (3HPG), a selective group I mGluR agonist. The differing results reported in the current study may be attributable to differences in experimental design, including the use of a different species as a basis for cultured cortical neurons. However, another explanation may relate to the presence or absence of Mg^{2+} in the extracellular solution. Our experiments were performed in the presence of 10 μM Mg^{2+}, as this ion is normally present *in vivo*, whereas Yu and colleagues utilized a nominally free Mg^{2+} extracellular solution. Recently, it has been reported that the group I mGluR-mediated potentiation of NMDA-induced currents in frog motoneurons requires a minimum Mg^{2+} concentration; no effect on these currents was observed in a nominally free Mg^{2+} extracellular solution (32). However, earlier studies have reported potentiation of NMDA receptor-mediated currents by ACPD in rat spinal dorsal horn neurons (10) and rat hippocampal slices (29) even in nominally free Mg^{2+} extracellular solution. Thus, the effects of Mg^{2+} on modulation of NMDA receptors by group I mGluR remains unclear and may depend on the cell type and preparation under study.

Studies using transfected human embryonic kidney (HEK) 293 cells, which lack native mGluR, demonstrated that the effects of group I mGluR stimulation on NMDA-induced currents depends upon the composition of the NMDA receptor complex and the concentrations of NMDA and glycine used (14). DHPG may directly inhibit, potentiate, or have no effect on NMDA-induced currents depending upon the NMDA subunits expressed (14). Direct potentiation of NMDA-induced currents by DHPG was only observed with reduced NMDA (50 μM) and glycine (100 nM) concentrations (14). It is unlikely that the potentiation reported in the present study is due to a direct action at the NMDA receptor as the concentrations of NMDA and glycine (200 and 20 μM, respectively) were similar to experimental conditions in which DHPG had no effect on or inhibited NMDA-induced currents in transfected HEK293 cells (14).

Nicoletti *et al.* (46) recently hypothesized that the variability in effects found with group I mGluR activation, at least in the setting of neuronal injury, may be due to differing subunits in the NMDA receptor complex. The composition of the NMDA receptor complex in the rat cortical neurons used in the present study was not studied and could conceivably differ from the composition found in the mouse cortical neurons used by Yu and colleagues. Regardless, both the calcium imaging and the electrophysiology studies presented here provide consistent evidence of group I mGluR-induced potentiation of NMDA currents in rat cortical neurons. These findings are consistent with the hypothesis that potentiation of NMDA receptors by group I mGluR may contribute to neuronal injury, at least in rat cortical neuronal cultures.

The mechanism whereby group I mGluR potentiate NMDA receptor-mediated currents is unknown. It has been reported in other cell types that this effect may be mediated through a PKC-dependent pathway (4, 49), although others have failed to block group I mGluR-mediated potentiation using PKC inhibitors (29, 50). Recently, it has been suggested that the mechanism may involve a calmodulin-dependent reduction of the Mg^{2+} blockade of NMDA receptors (32). Another possibility may relate to the release of arachidonic acid after stimulation of group I mGluR. Arachidonic acid also potentiates NMDA-generated currents (40). In the present study we demonstrate that activation of group I mGluR leads to arachidonic acid release, suggesting a possible alternative mechanism whereby group I mGluR may potentiate NMDA currents.

However, enhancement of NMDA receptor currents cannot be the sole mechanism whereby group I mGluR mediate injury. We have previously reported that the modulatory effects of group I mGluR activation/inhibition on *in vitro* mechanical trauma of neuronal–glial cultures persist in the presence of NMDA receptor blockade (43). In the present study, we were interested in whether this phenomenon was restricted to traumatic injury or would be a feature of other injurious stimuli. Similar to our results with traumatic injury, exacerbation of injury by group I mGluR activation or protection by group I mGluR inhibition in the presence of MK801 was also observed after OGD-induced injury. Thus, at least under conditions of NMDA receptor blockade, group I mGluR may act through other signal transduction pathways to exacerbate neuronal injury.

Nonetheless, these studies do not exclude the possibility that NMDA receptor potentiation may be an important mechanism for injury modulation by group I

mGluR in the absence of NMDA receptor blockade. The presence of MK801 may alter the characteristics of injury, as has been suggested for the OGD model of neuronal injury (27). It has also recently been reported that potentiation of NMDA-mediated currents by ACPD in frog motoneurons occurs even in the presence of open channel blockers such as MK801 (32). However, the dose of MK801 used here has been shown by us to completely block NMDA-induced cell death in neuronal–glial cultures and is 1 order of magnitude greater than the maximally effective dose against traumatic injury in these cultures (42, 43).

We have previously detailed a novel injury model, in which mechanical trauma is coupled with a brief period of "ischemic-like" conditions (2). This model may more accurately predict traumatic brain injury (TBI) *in vivo*, because TBI is frequently accompanied by ischemia (7, 38, 63). In addition, this model induces both necrotic cell death and caspase-dependent apoptotic cell death, which is similar to findings after *in vivo* TBI (2). Consistent with our results using OGD, mechanical trauma and *in vivo* TBI (3, 43), DHPG exacerbates, whereas AIDA inhibits, cell death following trauma + 3NP/GD.

Liberation of arachidonic acid occurs after *in vivo* trauma (15, 20, 34, 61) or following stimulation *in vitro* with glutamate analogues (52, 59). Phospholipid hydrolysis generates free radicals (15) and arachidonic acid or its metabolites may contribute to injury *in vivo* through a variety of mechanisms (16, 20, 33). In transfection studies using Chinese hamster ovary cells, activation of mGluR1 induces release of arachidonic acid (5). Arachidonic acid release also occurs in cultured neurons after stimulation with a nonselective mGluR agonist (19). However, the specific mGluR group involved in this arachidonic acid release is unknown. In the present study, we demonstrate selective activation of group I mGluR in neuronal–glial cultures releases [^{3}H]AA into the culture medium.

A direct coupling between group I mGluR and phospholipase A_2 (PLA_2) may not necessarily be the mechanism underlying DHPG-induced [^{3}H]AA release. Activation of NMDA receptors also leads to the liberation of arachidonic acid, possibly by increasing intracellular calcium levels that subsequently induce PLA_2 activity (18, 36). Thus, group I mGluR activation may result in release of arachidonic acid indirectly through potentiation of NMDA receptors or through release of calcium from intracellular stores.

Significant liberation of [^{3}H]AA occurred after trauma + 3NP/GD injury to neuronal–glial cultures, and this release was potentiated by administration of DHPG during trauma. Similar results were observed for OGD injury at 60 min; however, this model exhibited significantly more variability in [^{3}H]AA release (unpublished observations). In the present study, we were unable to successfully inhibit PLA_2 in our cultures due to the toxic effects of prolonged exposure to all of the PLA_2 inhibitors tested, including arachidonyltrifluoromethyl ketone ($AACOCF_3$), quinacrine, and 7,7-dimethyl-(5*Z*,8*Z*)-eicosadienoic acid (unpublished observations). Although the data presented here only support a correlation between increased injury-induced arachidonic acid release and cell death by DHPG, the findings are consistent with the conclusion that arachidonic acid release may contribute, at least in part, to group I mGluR-mediated injury.

As noted above, in addition to its other well-known toxic effects, arachidonic acid potentiates NMDA receptor-mediated currents (40). This suggests a possible mechanism whereby group I mGluR may exacerbate injury via increases in arachidonic acid release. Furthermore, potentiation of glutamate release by group I mGluR is most pronounced in the presence of arachidonic acid (30), allowing for a possible positive feedback loop, wherein group I mGluR stimulation releases arachidonic acid, which in turn increases glutamate release. Such glutamate release may serve to further activate both NMDA receptors and group I mGluR.

In summary, group I mGluR-mediated exacerbation of neuronal injury may occur as a result of activation of multiple parallel and possibly serial pathways. Potentiation of NMDA receptors by group I mGluR remains an attractive hypothesis because of the central role that NMDA receptors play in neuronal injury (21, 22, 55). However, the results presented here indicate that other signal transduction pathways may also be involved, such as release of arachidonic acid, and that these alternate pathways may become more important during therapeutic interventions such as blockade of NMDA receptors.

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Research report

Selective mGluR5 receptor antagonist or agonist provides neuroprotection in a rat model of focal cerebral ischemia

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Abstract

Activation of group I metabotropic glutamate receptors (mGluR) has been implicated in the pathophysiology of acute central nervous system injury. However, the relative roles of the two group I subtypes, mGluR1 or mGluR5, in such injury has not been well examined. We compared the effects of treatment with the newly developed, selective mGluR5 antagonist 2-methyl-6-phenylethynylpyridine (MPEP) and the selective mGluR5 agonist (*R,S*)-2-chloro-5-hydroxyphenylglycine (CHPG) in a rat intraluminal filament model of temporary middle cerebral artery occlusion (MCAo). Rats were administered MPEP or CHPG i.c.v. beginning 15 or 135 min after induction of ischemia for 2 h. Infarct size was measured after either 22 or 70 h of reperfusion, and neurological function was quantified at 2, 24, 48 and 72 h. Treatment with MPEP or CHPG at 15 min reduced 24 h infarct volume by 61 and 44%, respectively. The neuroprotective effects were dose dependent. Delaying MPEP treatment until 135 min eliminated the neuroprotective effects. In other studies, using early MPEP treatment (15 min) at optimal doses, infarct volume was reduced by 44% at 72 h and this was correlated with significant neurological recovery. These data suggest that both MPEP and CHPG are neuroprotective when administered after focal cerebral ischemia. In separate, recent studies we found that although MPEP does act as an mGluR5 antagonist and blocks agonist induced phosphoinositide hydrolysis, it also serves as a non-competitive NMDA antagonist; in contrast, other results indicate that CHPG mediated neuroprotection may reflect anti-apoptotic activity. Therefore, both types of compounds may prove to have therapeutic potential for the treatment of stroke.

Theme: Disorders of the nervous system

Topic: Ischemia

Keywords: Cerebral ischemia; Neuroprotection; mGluR5 receptor; Non-competitive antagonist

1. Introduction

Glutamate, a major excitatory neurotransmitter in the mammalian central nervous system, interacts with both ionotropic glutamate receptors and metabotropic glutamate receptors (mGluR). A role for ionotropic glutamate receptors in neuronal cell death following cerebral ischemia is well established [8,12]. Because such receptors play such a critical role in fast synaptic transmission, blockade of these receptors may be associated with substantial side effects. Recent evidence suggests that mGluR, which are G-protein coupled receptors, may provide an effective alternative approach for reducing glutamate mediated cell death. Furthermore, the fact that mGluRs are primarily localized in the CNS may serve to limit certain peripheral side effects [9,20]. It should also be noted that blockade of mGluRs seems to have only a modest impact on fast excitatory transmission [7].

There are eight mGluR subtypes, which have been divided into three major groups on the basis of sequence homology, signal transduction pathways, and pharmacological sensitivities [20,21]. Group I mGluR includes mGluR1 and mGluR5; activation of these receptors causes stimulation of phospholipase C, resulting in phosphoinositide (PI) hydrolysis and intracellular calcium mobilization [20,21]. The role of group I mGluR in

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neurodegeneration remains controversial. Whereas antagonists of these receptors are consistently neuroprotective, agonists have been found to either amplify or attenuate neuronal cell death [16]. Previous studies have suggested that the neurotoxic effects of putative group I ligands may be modulated primarily by the mGluR1 [6,14,15], but until recently, selective subtype specific antagonists have not been available to address this issue. The potent and selective mGluR1 antagonists (*S*)-4-carboxypheylglycine (AIDA) reduces traumatic neuronal injury in vivo and in vitro, and attenuates the delayed degeneration of vulnerable neurons in gerbils subjected to transient global ischemia [6,19]. In addition, the selective mGluR1a antagonists (+)-2-methyl-4-carboxyphenylglycine (LY367385) and 7-hydroxyiminoclopropan[*b*]chromen-1a-carboxylic acid ethyl ester (CPCCOEt) have recently been described as being neuroprotective [2,6]. From these studies it may be concluded that activation of mGluR1a receptors contributes to glutamate neurotoxicity and post-ischemic cell death, conditions that largely reflect necrotic cell death. In contrast to mGluR1, mGluR5 activation may serve to attenuate apoptotic cell death [3,4]. This conclusion was partly based on studies using antisense oligonucleotides directed at mGluR5 in cerebellar granule cells cultures subjected to low levels of potassium [4]. In addition, activation of mGluR5 protects cultured granule cells against apoptotic death [1,4].

Recent development of more selective mGluR1 and mGluR5 agonists/antagonists has provided tools to further address such hypotheses. These include the mGluR5 agonist (*R,S*)-2-chloro-5-hydroxyphenylglycine (CHPG) and antagonist 6-methyl-2-(phenylethynyl)-pyridine (MPEP) [5,7]. CHPG has been shown to activate only mGluR5, but not mGluR1, in transfected cells; in vitro it has been shown to reduce neuronal apoptosis [5]. MPEP has been described as a selective non-competitive mGluR5 antagonist with no appreciable agonist or antagonist activity at recombinant mGlu1b, group II or III mGluRs [7]. Furthermore, MPEP does not act at the extracellular glutamate binding site of mGluR5 receptors common to all known competitive mGluR antagonists. Rather it interacts with transmembrane domains III and VII of mGluR5 receptors [18], which makes MPEP less sensitive to the ambient concentration of glutamate. In the present study, we compared the effects of MPEP or CHPG treatment in a rat intraluminal filament model of temporary middle cerebral artery occlusion (MCAo).

2. Materials and methods

2.1. Surgical procedures

Male Sprague–Dawley rats (260–300 g; Charles River Lab., Raleigh, VA, USA) were used in this study. Anesthesia was induced by 5% halothane and maintained at 2% halothane delivered in oxygen. Body temperature was maintained normothermic (37±1°C) throughout all surgical procedures by means of a homeothermic heating system (Harvard Apparatus, South Natick, MA, USA). Food and water were provided ad libitum before and after surgery, and the animals were individually housed under a 12-h light–dark cycle. The facilities in which the animals were maintained and fully accredited by the American Association for the Accreditation of Laboratory Animal Care (AALAC). In conducting the research described in this report, the investigators adhered to the Guide for Care and Use of Laboratory Animals, as promulgated by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Resources, National Research Council.

The i.c.v. catheters were stereotaxically placed into the right lateral ventricle (1.5 mm posterior and 1 mm right lateral to bregma). Two cortical electrodes (epidural stainless steel screw electrodes, 0-80×1/8 in.) were permanently implanted and fixed to the skull using dental acrylate cement [23]. At 72 h after the surgical procedure described above, rats were reanesthetized and prepared for temporary focal ischemia using the filament method of middle cerebral artery occlusion (MCAo) and reperfusion as described elsewhere [22,24]. Briefly, the right external carotid artery was isolated and its branches were coagulated. A 3-0 uncoated monofilament nylon suture with a rounded tip was introduced into the internal carotid artery via the external carotid artery and advanced (approximately 22 mm from the carotid bifurcation) until a slight resistance was observed, thus occluding the origin of the MCA. Once the filament was in place, a drop in amplitude of the cortical electroencephalographic (EEG) recording was used to indicate a successful occlusion. The endovascular suture remained in place for 2 h and then was retracted to allow reperfusion of the MCA. After surgery, animals were placed in recovery cages with ambient temperature maintained at 22°C.

Prior to MCAo, the body temperature was recorded and a neurological examination was performed (see below). EEG activity was measured in each rat under anesthesia immediately before MCAo, immediately before reperfusion at 2 h after occlusion, and again at the 24- or 72-h endpoint before euthanasia. This enabled us to establish an experimental exclusion criterion (i.e. diminution in EEG amplitude by 70% or greater at 2 h after occlusion compared with preocclusion amplitude) and to determine the effect of MPEP or CHPG on cortical EEG activity in injured rats. This exclusion criterion has been validated by us previously and used in multiple prior studies that examine neuroprotective drugs treatments [22–24]. Changes in EEG amplitude were quantified with the use of spectral analysis and data reported as percent EEG recovery compared with the 2-h reperfusion sample. At the end of each experiment (either 24 or 72 h), rats were euthanized by decapitation and their brains were removed for quantification of infarction.

2.2. Neurological examination

A neurological examination was performed on each rat immediately before MCAo, before reperfusion, and at 24, 48 and 72 h after MCAo. Neurological scores were derived using a 10-point ordinal scale. Each animal was examined for reduced resistance to lateral push (score=4), open field circling (score=3), and shoulder adduction (score=2) or contralateral forelimb flexion (score=1) when held by the tail [22,24]. Rats extending both forelimbs toward the floor and not showing any other signs of neurological impairment were scored 0. Using this procedure, maximal neurological severity was measured as a cumulative score of 10. In the present study all rats subjected to MCAo either exhibited a neurological score of 10 when examined 2 h after ischemia or immediately before reperfusion, or were excluded from the study (two of the total experimental rats).

2.3. Infarct analysis

For each rat brain, analysis of ischemic cerebral damage was performed, including total and core infarct volumes and hemispheric infarct size (calculated as percentage of infarcted tissue referenced to the corresponding contralateral uninjured cerebral hemisphere, using 2,3,5-triphenyltetrazolium chloride (TTC) staining from seven coronal sections (2-mm thick). Brain sections were taken from the region beginning 1 mm from the frontal pole and ending just rostral to the corticocerebellar junction. Computer-assisted image analysis was used to calculate infarct volumes, described in detail elsewhere [22,24]. Briefly, the posterior surface of each TTC-stained forebrain section was digitally imaged (Loats, Westminster, MD, USA) and quantified for areas (in square millimeters) of ischemic damage. Core injury was defined as brain tissue completely lacking TTC staining, whereas total injury was specified as all ipsilateral tissue showing a loss of stain compared with the contralateral, uninjured hemisphere. Sequential integration of the respective areas yielded total and core infarct volumes (in cubic millimeters). Similarly, ipsilateral and contralateral hemispheric volumes were measured where hemispheric swelling (edema) was expressed as the percentage increase in size of the ipsilateral (occluded) hemisphere over the contralateral (uninjured) hemisphere. Penumbral areas were defined as the total (green outline) minus the core (yellow outline) infarct volume, which correlated to light pink-staining brain regions (Fig. 1).

2.4. Data analysis

Data are presented as mean±S.E.M. Statistical analysis of single dose responses (n=7–10/group) was made by comparisons using independent Student's t test with a modified Bonferroni correction for multiple comparisons.

2.5. Compound and treatment

MPEP was dissolved in DMSO and diluted in saline (1% final DMSO concentration). CHPG was dissolved in saline. CHPG, MPEP or vehicle (saline or DMSO 1%) were administered i.c.v. in a 5-μl volume. All injections were given 15 min after occlusion. For the 24-h recovery studies vehicle (n=11), MPEP or CHPG (25–250 nmol, n=8–9 per dose) were injected. For the 72-h experiments vehicle (n=8) or MPEP (250 nmol, n=7) was studied. In other experiments, the injection of MPEP was delayed until 135 min after MCAo.

3. Results

3.1. 2-h MCAo in vehicle-treated rats

MCAo with 22 h reperfusion resulted in significant core infarction within the temporal/parietal cortex and underlying striatum of the ipsilateral (injured) hemisphere. Ischemic damage generally extended from the most rostral forebrain sections to the final caudal sections and was greatest in the area around the bregma (Fig. 1). Total and core infarct volumes averaged 303±17 and 199±14 mm^3, respectively. At 2 h after MCAo, neurological function (10.0±0.0) and EEG activity were markedly reduced. Neuroscores at 24 h after MCAo exhibited a significant degree of spontaneous recovery to (6.9±0.7) (Table 1).

At 72 h after MCAo, the mean total and core infarct volume (364±24 and 247±17 mm^3, respectively) were larger than those observed at 24 h. However, neurological function showed additional and significant spontaneous improvements (2.6±0.4) (Table 2).

3.2. Physiological parameters

All MCAo animals lost approximately to 10–16% of body weight during the 24 h recovery period (22–27% loss at 72 h), regardless of treatment group, with no significant difference in body weight loss between groups. In vehicle-treated animals, MCAo was associated with a transient, mild increase in body temperature (38.0±0.4°C), which returned to normal by 4–6 h post occlusion. Interestingly, at 72 h post injury, control injured animals exhibited a mild hypothermia (35.3±0.4°C). At all time points, temperature measurements from MPEP or CHPG treated animals were not significantly different from those of the corresponding vehicle-treated animals.

3.3. Effect of early treatment of MPEP or CHPG on neuroprotection

Both MPEP and CHPG reduced the core infarct volume when administered 15 min after occlusion, as shown in Fig. 1. Although all doses of MPEP and CHPG reduced core infarct volume, only the highest dose (250 nmol)

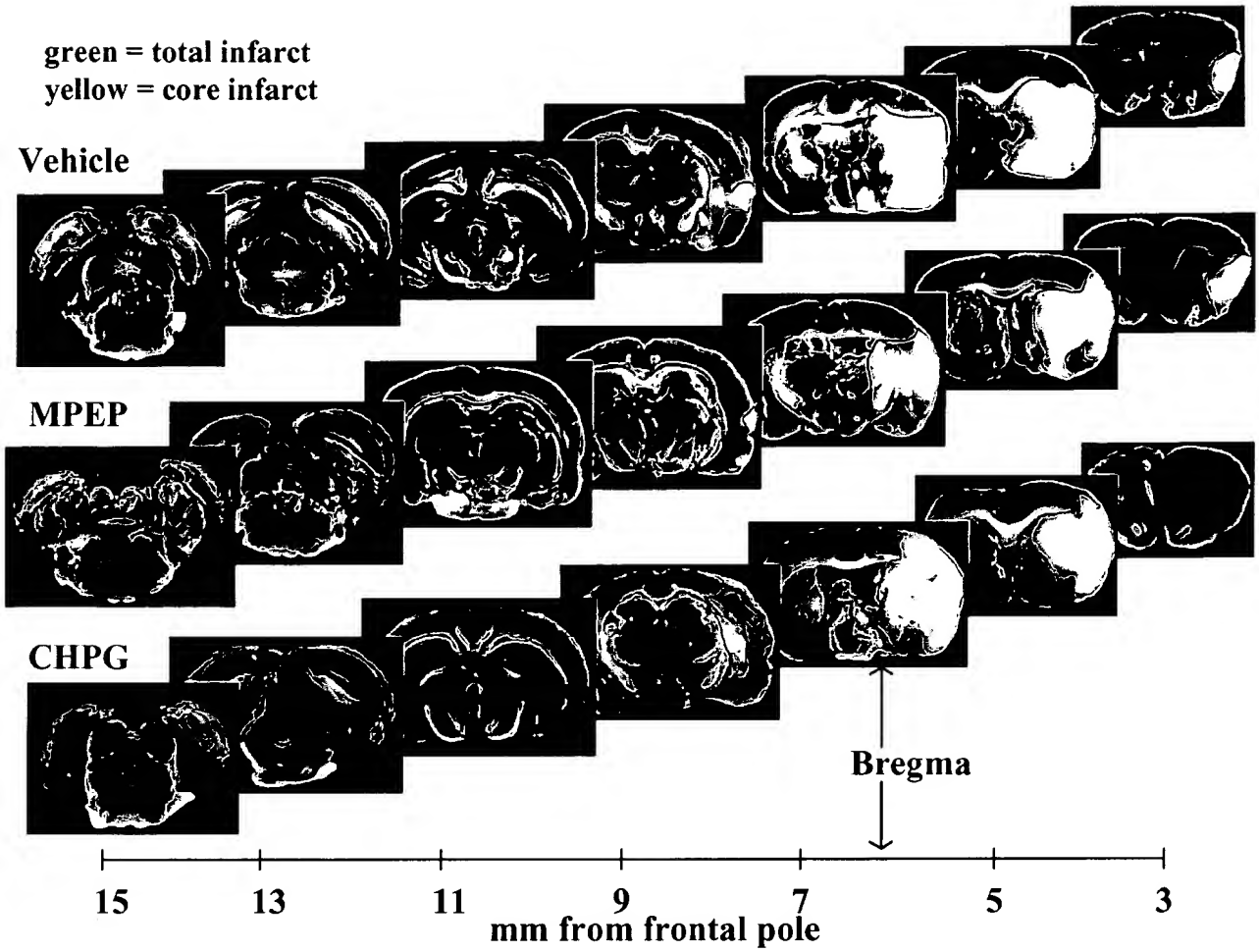

Fig. 1. Representative forebrain images of vehicle versus MPEP or CHPG (250 nmol, i.c.v.) administration after 2 h MCAo and a 22-h reperfusion. Brain sections were stained with TTC, and total infarct volume (green outline) and core infarct volume (yellow outline) were defined.

produced a significant reduction infarct volume to 74±14 mm^3 (MPEP) or 104±34 mm^3 (CHPG) (Fig. 2). Normalization of the MPEP and CHPG infarct data to vehicle-treated rats established that at highest dose tested the neuroprotection was 61% (MPEP) and 44% (CHPG), respectively. Examination of neurological function at 24 h in MPEP or CHPG-treated rats showed a trend toward improved neurological function, which did not reach significance (Table 1).

In separate experiments, the neuroprotective actions of

Table 1
Effect of MPEP and CHPG (administered i.c.v.) on core neuroprotection, EEG recovery and neurological function at 24 h after MCAo/reperfusion

Treatment	Dose (nmol)	*n*	Neuroprotection[a] (%)	EEG recovery[b] (%)	Neurological score
Vehicle		36	0	26±11	6.9±0.7
MPEP	25	9	14±14	30±13	4.8±0.5
	75	8	28±9	66±18	5.0±0.8
	250	8	60±8**	50±21	4.9±2.8
CHPG	25	8	20±6	41±28	5.9±2.6
	75	7	22±11	31±15	5.9±0.7
	250	8	44±14*	40±20	6.8±0.9

Values are presented as mean±S.E.M.; *, $P<0.05$, **, $P<0.01$, compared with vehicle-treated group (independent Student's *t* test with a modified Bonferroni correction for multiple comparisons).

[a] Percentage reduction of infarct volume after treatment compared with vehicle-treated group.

[b] Percentage recovery of EEG power compared with pre-MCAo recording.

Table 2
Effect of MPEP and CHPG (250 nmol, i.c.v.) on core neuroprotection, EEG recovery and neurological function at 72 h after MCAo/reperfusion

Treatment	n	Neuroprotection[a] (%)	EEG recovery[b] (%)	Neurological score			
				2 h	24 h	48 h	72 h
Vehicle	6	0	9±6	9.5±0.5	7.2±1.4	3.0±0	2.6±0.4
MPEP	8	44±5**	20±8	10.0±0	5.0±0.8*	2.8±0.3	1.4±0.4*
CHPG	7	26±13	32±11	10.0±0	6.2±0.9	4.7±0.8	3.0±1.1

Values are presented as mean±S.E.M.; *, $P<0.05$, **, $P<0.01$, compared with vehicle-treated group (independent Student's t test with a modified Bonferroni correction for multiple comparison).
[a] Percentage reduction of infarct volume after treatment compared with vehicle-treated group.
[b] Percentage recovery of EEG power compared with pre-MCAo recording.

MPEP and CHPG (250 nmol) were examined at 72 h. MPEP-treated rats had a reduction in total and core infarct volumes to 245±22 and 138±11 mm^3, respectively (Fig. 3), corresponding to a significant neuroprotection in both brain areas (total=33±6%, core=44±5%). Neurological recovery (1.4±0.4) was also significantly improved at 72 h (Table 2). CHPG-treated rats had a reduction the total and core infarct volumes to 301±31 and 184±40 mm^3 respectively (Fig. 3), but these changes did not reach significance.

3.4. Effect of delayed MPEP treatment on percent neuroprotection at 24 h

The effect of delaying treatment with 250 nmol MPEP was also examined. When rats were treated at 15 min after reperfusion (or 135 min postinjury), limited reductions in infarct volumes (20±8%) and neuroscores (5.0±0.8) were observed, which did not reach significance (Fig. 4), in contrast to the significant effects observed with treatment at 15 min post-occlusion.

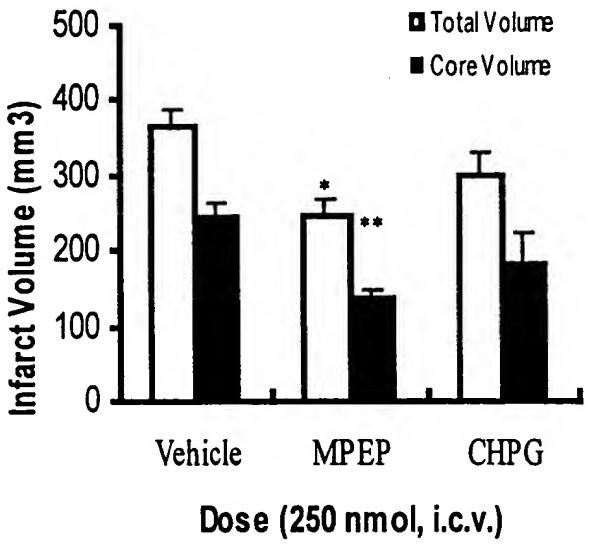

Fig. 3. Effects of MPEP or CHPG (250 nmol, i.c.v.) treatment on infarct volume at 72 h after temporary MCAo. Data are presented as mean±S.E. *, $P<0.05$, **, $P<0.01$ compared with vehicle-treated group (independent Student's t test with a modified Bonferroni correction for multiple comparisons).

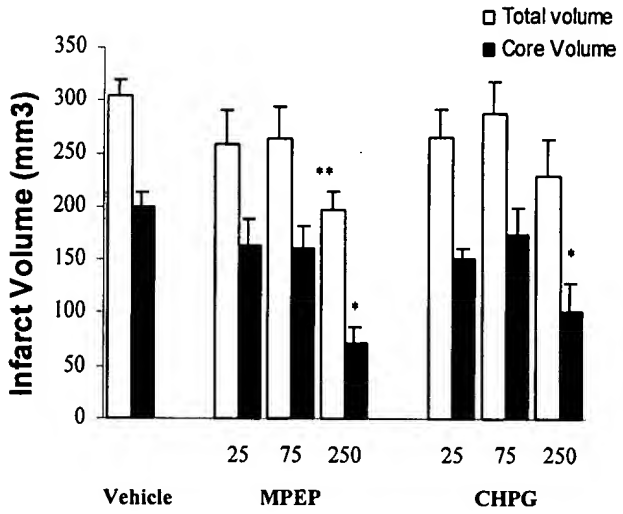

Fig. 2. Effects of increasing dose of MPEP or CHPG (nmol, i.c.v.) on infarct volume at 24 h after temporary MCAo. Data presented as mean±S.E. *, $P<0.05$, **, $P<0.01$ compared with vehicle-treated group (independent Student's t test with a modified Bonferroni correction for multiple comparisons).

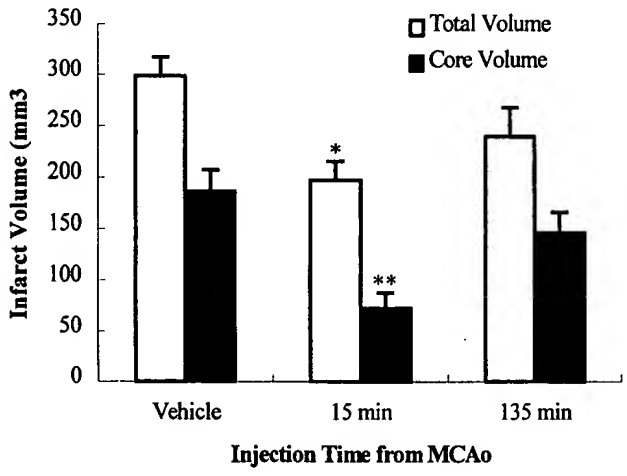

Fig. 4. Compares effect of early or delayed treatment (from time of MCAo) with MPEP (250 nmol, i.c.v.) on infarct volume at 24 h after temporary MCAo. Data are presented as mean±S.E. *, $P<0.05$, **, $P<0.01$ compared with vehicle-treated group (independent Student's t test with a modified Bonferroni correction for multiple comparisons).

4. Discussion

The intraluminal filament model of MCAo used in this study produces ischemia through temporary occlusion of the MCA with subsequent reperfusion at controlled time points. We have shown that both the selective mGluR5 agonist (CHPG) and antagonist (MPEP) have neuroprotective effects in this model. However, the neuroprotective mechanisms are likely to be different. Our in vivo observations are supported by in vitro results, which also showed that treatment with CHPG protects against apoptotic cell death [1]. Based on the in vitro results, it was suggested that CHPG may provide neuroprotection by limiting neuronal apoptosis after cerebral ischemia. Although we did not distinguish necrosis and apoptosis in our experiments, it has been reported that both can occur in response to focal cerebral ischemia [10,11].

Interestingly, our findings also demonstrated that the selective mGluR5 antagonist MPEP significantly reduces neuronal damage and improves neurological recovery induced by focal cerebral ischemia. These observations are also consistent with recent in vitro and in vivo results, which showed that MPEP and the structurally related selective mGluR5 antagonist SIB-1893 significantly attenuate post traumatic neuronal cell death and improve functional recovery [13].

Experiments from our laboratory suggest that the protective effects of MPEP may reflect non-competitive antagonism of the NMDA receptor, rather than through actions at mGluR5. For example, whereas antisense directed against mGluR1 or mGluR5 equally reduced group I mGluR-mediated increases in PI hydrolysis accumulation, only antisense against mGluR1 significantly attenuated traumatic neuronal injury in an in vitro model of necrotic cell death [14]. In addition, although we found that MPEP acts as an effective mGluR5 antagonist in rat cortical neuronal cultures, as shown by complete inhibition of PI hydrolysis induced by CHPG, the concentration of MPEP required to block agonist-induced PI hydrolysis was 100 times lower than that required for neuroprotection [13]. The noncompetitive NMDA receptor antagonist, (5*R*,10*S*)-(+)5-methyl-10,11-dihydro-5H-dibenzo[*a*,*d*]cyclohepten-5,10-imine (MK801) markedly reduces glutamate or NMDA mediated neuronal cell death in vitro; however, no additive neuroprotective effect was observed when MPEP was applied to injured cells in presence of MK801 in our in vitro neurotoxic models or after mechanical injury [13]. In contrast, such additive neuroprotective effects have been shown using the mGluR1 antagonist AIDA in combination with MK801 [14]. Finally, MPEP significantly reduces steady state NMDA evoked whole-cell current at concentrations that are neuroprotective in cultured cortical neuronal cells, and also reduces the open time of the NMDA channel and the open probability of the channel [17]. This latter observation strongly suggests that MPEP acts as a non-competitive NMDA receptor antagonist.

In conclusion, as evidenced by lesion volume measurements, we have shown that both MPEP and CHPG provide neuroprotection when administered after focal cerebral ischemia. Based on in vitro studies, we suggest that a possible mechanism of action of CHPG may involve attenuation of apoptotic cell death, whereas MPEP appears to act as non-competitive NMDA receptor antagonist. Therefore, both types of compounds may prove to have therapeutic potential for the treatment of stroke.

Acknowledgements

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Altered synaptic plasticity in a mouse model of fragile X mental retardation

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Fragile X syndrome, the most common inherited form of human mental retardation, is caused by mutations of the *Fmr1* gene that encodes the fragile X mental retardation protein (FMRP). Biochemical evidence indicates that FMRP binds a subset of mRNAs and acts as a regulator of translation. However, the consequences of FMRP loss on neuronal function in mammals remain unknown. Here we show that a form of protein synthesis-dependent synaptic plasticity, long-term depression triggered by activation of metabotropic glutamate receptors, is selectively enhanced in the hippocampus of mutant mice lacking FMRP. This finding indicates that FMRP plays an important functional role in regulating activity-dependent synaptic plasticity in the brain and suggests new therapeutic approaches for fragile X syndrome.

Fragile X syndrome is a prevalent form of inherited mental retardation, occurring with a frequency of 1 in 4,000 males and 1 in 8,000 females. The syndrome is also characterized by developmental delay, hyperactivity, attention deficit disorder, and autistic-like behaviors (1). There is no effective treatment for fragile X syndrome.

The syndrome is typically caused by a repeat expansion mutation in the *FMR1* gene that encodes FMRP, the fragile X mental retardation protein. FMRP is known to associate with translating polyribosomes and a subset of brain mRNAs and is believed to function as a regulator of protein synthesis (2–5). Involvement of FMRP in synaptic plasticity has long been suspected, because polyribosomes, *FMR1* mRNA, and FMRP are all present in dendritic spines, the major site of synaptic transmission on cortical neurons (6). The *Fmr1* null mutant (knockout) (*Fmr1*-KO) mouse, which has a behavioral phenotype consistent with fragile X syndrome, provided an opportunity to test this hypothesis. However, protein synthesis-dependent late-phase long-term synaptic potentiation (LTP) was found to be unaffected in the hippocampus of mutant mice (7, 8).

Reexamination of this issue was prompted by recent work showing that local synaptic control of protein synthesis is required for stable expression of a second form of hippocampal synaptic modification: long-term depression (LTD) triggered by activation of group 1 metabotropic glutamate receptors (mGluRs) (9–11). A role for FMRP is this form of synaptic plasticity was further suggested by the fact that FMRP is one of the proteins known to be synthesized in response to mGluR activation (6).

We report here that mGluR-dependent LTD (mGluR-LTD) is significantly altered in the hippocampus of *Fmr1*-KO mice. Rather than a deficit, however, we find that mGluR-LTD is augmented in the absence of FMRP. This finding is consistent with the recent discovery that FMRP normally functions as a negative regulator of translation (5, 12, 13). We propose that exaggerated LTD and/or mGluR function are responsible for aspects of the behavioral phenotype in fragile X syndrome, and that antagonists of group 1 mGluRs should be considered as possible therapeutic agents.

Materials and Methods

Hippocampal slices were prepared from postnatal day (P)21–30, C57BL/6 congenic *Fmr1*-KO mice and their wild-type (WT) littermates, as described (10). Slices were collected in ice-cold dissection buffer containing (in mM): sucrose, 212; KCl, 2.6; NaH_2PO_4, 1.25; $NaHCO_3$, 26; $MgCl_2$, 5; $CaCl_2$, 0.5; and dextrose, 10. CA3 was removed immediately after sectioning. Slices recovered for 1–5 h at 30°C in artificial cerebrospinal fluid (ACSF) containing (in mM) NaCl, 124; KCl, 5; NaH_2PO_4, 1.25; $NaHCO_3$, 26; $MgCl_2$, 1; $CaCl_2$, 2; and dextrose, 10, saturated with 95% O_2, 5% CO_2. For recording, slices were placed in a submersion recording chamber and perfused with 30°C ACSF at a rate of 2 ml/min.

Field potentials (FPs) were recorded with extracellular recording electrodes (1.0 MΩ) filled with ACSF and placed in stratum radiatum of area CA1. Synaptic responses were evoked by a 200-μsec current pulse to Schaffer collateral axons with a concentric bipolar tungsten stimulating electrode. Stable baseline responses were collected every 30 sec by using a stimulation intensity (10–30 μA) yielding 50–60% of the maximal response. mGluR-LTD was induced in the presence of the *N*-methyl-D-aspartate (NMDA) receptor (NMDAR) antagonist D-(-)-2-amino-5-phosphono-pentanoic acid (D-APV) (50 μM) by using paired-pulse low-frequency stimulation (PP-LFS) consisting of 900 pairs of stimuli (50-msec interstimulus interval) delivered at 1 Hz. NMDAR-LTD was induced by using 900 single pulses delivered at 1 Hz (14).

Waveforms were filtered at 2 kHz, acquired, and digitized at 10 kHz on a personal computer by using EXPERIMENTER'S WORKBENCH (DataWave Systems, Boulder, CO). The group data were analyzed as follows: (*i*) the initial slope of the FP for each experiment was expressed as percentages of the preconditioning or 3,5-dihydroxyphenylglycine (DHPG) baseline average (2), and the time scale in each experiment was converted to time from the onset of conditioning or DHPG. All experiments were performed blind to the genotype of the mice, determined after analysis of individual experiments. After genotyping, the time-matched normalized data were averaged across experiments and expressed in the text and figures as the means (± SEM). Significant differences between groups were determined by using an independent *t* test and the Komolgarov–Smirnov test.

R,S-DHPG and D-APV were purchased from Tocris (St. Louis, MO). All other chemicals were purchased from Sigma. DHPG was prepared as a 100× stock in H_20, aliquoted, and stored at −20°C. Fresh stocks were made once per week. A 10× stock of D-APV was prepared in ACSF and stored at 4°C. These stocks were diluted in ACSF to achieve their final concentrations.

Abbreviations: LTP, long-term potentiation; mGluR, group 1 metabotropic glutamate receptor; LTD, long-term depression; mGluR-LTD, mGluR-dependent LTD; P*n*, postnatal day *n*; ACSF, artificial cerebrospinal fluid; FP, field potential; PP-LFS, paired-pulse low-frequency stimulation; DHPG, 3,5-dihydroxyphenylglycine; D-APV, D-(-)-2-amino-5-phosphono-pentanoic acid; *Fmr1*-KO, *Fmr1* knockout; WT, wild type; NMDA, *N*-methyl-D-aspartate; NMDAR, NMDA receptor; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid.

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Results

Normal Synaptic Transmission in *Fmr1*-KO Mice. Hippocampal slices were prepared from P21–30 C57BL/6 congenic *Fmr1*-KO mice and their WT littermates. Excitatory synaptic FPs evoked by stimulation of the Schaffer collaterals were recorded extracellularly from the stratum radiatum of area CA1. In all cases, the experimenters were blind to the genotype.

Previous studies have examined the properties of transmission at Schaffer collateral synapses in the CA1 region of hippocampus of these mutant mice. In terms of basal transmission, excitability, paired-pulse facilitation, early-phase LTP elicited with 100 Hz stimulation, and late-phase (protein synthesis-dependent) LTP induced with θ-burst stimulation, *Fmr1*-KO mice were indistinguishable from WT littermates (7, 8). It can be inferred from these findings that excitatory synaptic transmission mediated by α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and NMDARs and the state of inhibition are not appreciably affected by the absence of FMRP. Because of this extensive prior characterization, we did not examine these properties here. However, we did confirm that FP amplitudes in response to increasing stimulus current were not different between *Fmr1*-KO and WT littermates [$F(1,350) = 0.358, P > 0.5$], the maximum amplitude of FP from *Fmr1*-KO mice (1.73 ± 0.19 mV; $n = 39$ slices from 17 mice) was not different from WT (1.63 ± 0.16 mV; $n = 36$ slices from 18 mice; $P = 0.36$), and the stimulus currents used to evoke baseline responses were not different between groups (*Fmr1*-KO 22 ± 1 μA; WT 23 ± 2 μA).

mGluR-LTD Induced by Synaptic Stimulation Is Enhanced in *Fmr1*-KO Mice. Paired-pulse stimulation repeated at 1 Hz for 15 min (PP-LFS) induces LTD that is independent of NMDARs and requires activation of group 1 mGluRs (9, 10, 15) and the rapid translation of preexisting mRNA (9). We therefore first examined the consequences of PP-LFS in slices from KO and WT animals.

We found that PP-LFS (delivered in the presence of 50 μM D-APV to block NMDARs) produced a small but significant LTD in WT mice ($93 \pm 3\%$ 60 min after PP-LFS; $n = 21$ slices from 10 mice; Fig. 1). The magnitude of LTD in these experiments is considerably less than what we have seen in previous studies using rats (9, 10). This difference is likely because of the species and the strain of mice used. This finding of reduced LTD in C57BL/6 mice was not entirely unexpected, as the magnitude of the NMDAR-dependent form of LTD is also typically less in these animals as compared with rats (personal observations). However, we were surprised to find that the magnitude of LTD induced with PP-LFS was significantly increased in slices prepared from KO animals ($82 \pm 3\%$; $n = 18$ slices from 8 mice; different from WT at $P < 0.004$; t test). The difference first emerged approximately 15 min after the tetanus; there was no indication that responses during or immediately after the PP-LFS were different in KO and WT animals (Fig. 1*A*). To further evaluate whether the distribution of depression values was different between KO and WT groups, a Kolmolgarov–Smirnov test was performed on the cumulative probability distribution, and this confirmed a significant difference ($P < 0.05$; Fig. 1*B*).

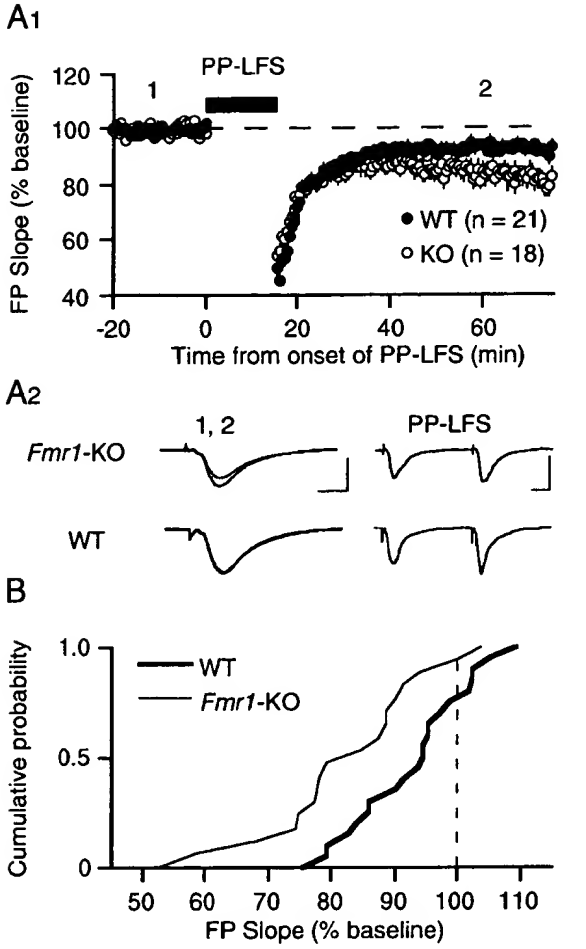

Fig. 1. Synaptic induction of mGluR-LTD using PP-LFS is significantly enhanced in hippocampus of *Fmr1*-KO mice as compared with WT controls. (*A1*) Average time course of the change in FPs after PP-LFS. LTD in KO animals measured $82 \pm 3\%$ of prePP-LFS baseline ($n = 18$ slices from 8 mice; open circles) as compared with $93 \pm 2\%$ in WT controls ($n = 21$ slices from 10 mice; filled circles; different at $P = 0.004$, t test). (*A2*) Representative FPs (2 min average) taken at the times indicated by the numbers on the graph. [Bars = 1 mV, 5 msec (1, 2) and 1 mV, 10 msec (PP-LFS).] (*B*) Cumulative probability distributions of FP slope values (% of baseline), measured 1 h after PP-LFS in individual slices from both KO and WT groups. The distribution in KO mice is significantly different from that in WT mice, as determined by Kolmolgarov–Smirnov test ($P < 0.05$). All experiments were performed blind, in the presence of the NMDAR antagonist D-APV (50 μM).

mGluR-LTD Induced by DHPG Is Enhanced in *Fmr1*-KO Mice. Another way to induce mGluR LTD is to apply the selective group 1 mGluR agonist DHPG (16). The advantages of this approach are that more synapses are affected more uniformly, and that it circumvents the need for presynaptic activation. Previous work under the same conditions as our experiments has shown a dose-dependent induction of LTD after DHPG (50–100 μM, 5 min). Activation of mGluR5 is required for induction, and protein synthesis is required for stable expression, of DHPG-LTD (9, 10). LTD with PP-LFS and DHPG are also mutually occluding, suggesting they use the same saturable expression mechanism (10). Therefore, in an attempt to confirm that mGluR-LTD is increased in *Fmr1*-KO mice using an independent method, we performed another series of experiments using DHPG to induce plasticity.

As in the previous study, experiments were performed in the presence of D-APV to eliminate the confound of NMDAR-dependent synaptic modifications. We used 100 μM DHPG (5 min) to induce a saturating level of LTD. The results showed, again, a significant enhancement of mGluR-LTD in slices from KO mice (Fig. 2). DHPG application to slices from *Fmr1*-KO mice resulted in depression of FP slope values to $77 \pm 3\%$ of preDHPG baseline (measured 60 min after DHPG application $n = 21$ slices from 9 mice). In comparison, DHPG-induced LTD was $88 \pm 4\%$ in WT mice (15 slices from 8 animals; $P = 0.02$;

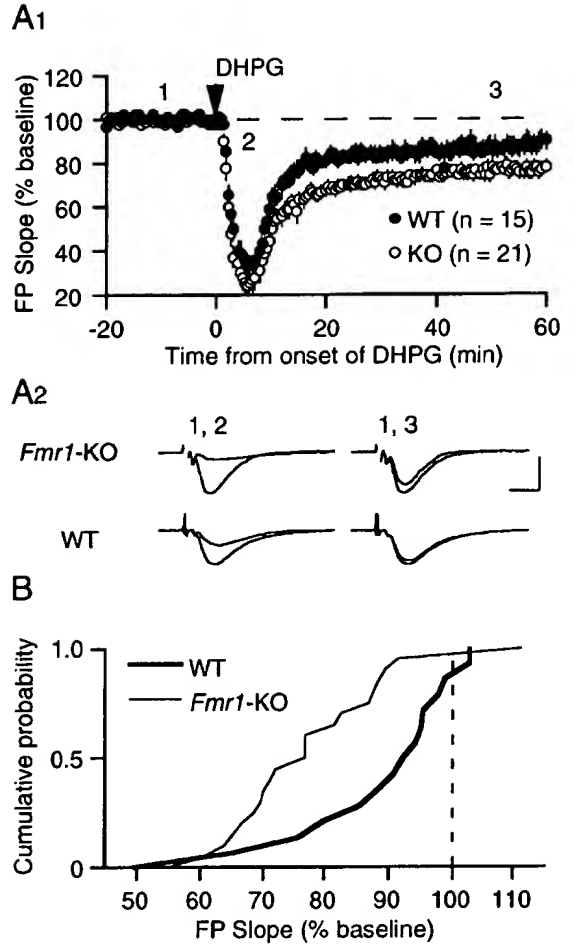

Fig. 2. Brief application of the mGluR agonist DHPG (5 min; 100 μM) induces greater LTD of synaptic responses in hippocampus of *Fmr1*-KO mice as compared with WT littermate controls. (*A1*) Plotted are average (±SEM) FP slope values over the time course of the experiment. In *Fmr1*-KO animals, the response 60 min after treatment was depressed to 77 ± 3% of preDHPG baseline (n = 21 slices from 9 mice; open circles); in interleaved WT controls, the response was depressed to 88 ± 4% of baseline (n = 15 slices from 8 mice; filled circles; different at P = 0.02; t test). (*A2*) Representative FPs (2 min average) taken at the times indicated by the numbers on the graph. (Bar = 1 mV; 5 msec.) (*B*) Cumulative probability distributions of FP slope values (% of baseline), measured 1 h after DHPG in individual slices from both KO and WT groups. The distribution in KO mice is significantly different from that in WT mice as determined by Kolmolgarov–Smirnov test ($P < 0.05$).

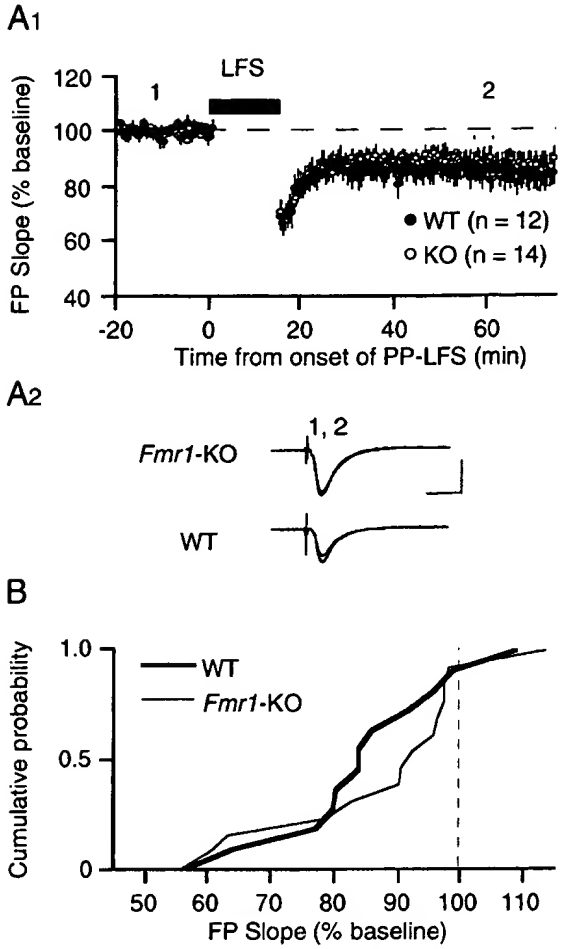

Fig. 3. Synaptic induction of NMDAR-dependent LTD using a 1-Hz LFS protocol is comparable in *Fmr1*-KO mice and WT controls. (*A1*) Average time course of the change in FPs after LFS. LTD in KO animals measured 86 ± 4% of preLFS baseline (n = 14 slices from 8 mice; open circles) as compared with 84 ± 4% in WT controls (n = 12 slices from 4 mice; filled circles; P = 0.6, t test). (*A2*) Representative FPs (2 min average) taken at the times indicated by the numbers on the graph. (Bar = 1 mV, 10 msec.) (*B*) Cumulative probability distributions of FP slope values (% of baseline), measured 1 h after LFS in individual slices from both KO and WT groups. The distribution in KO mice is not significantly different from that in WT mice as determined by the Kolmolgarov–Smirnov test. All experiments were performed blind.

Fig. 2*A*). The Kolmolgarov–Smirnov test performed on the cumulative probability distribution confirmed the statistical significance of this difference ($P < 0.05$; Fig. 2*B*). Although the acute effect of DHPG on synaptic transmission also appeared to be slightly enhanced in *Fmr1*-KO slices, this difference was not statistically significant (maximal acute depression: WT: 36 ± 4%, KO: 26 ± 5% of preDHPG baseline values). Western blots of hippocampal homogenates also confirmed that mGluR5 levels are comparable in KO and WT mice (data not shown).

NMDAR-Dependent LTD Is Normal in *Fmr1*-KO Mice. Two forms of homosynaptic LTD coexist at CA3–CA1 synapses: mGluR-LTD and a form that is triggered by activation of NMDARs (NMDAR-LTD) (17). NMDAR-LTD in hippocampal slices is independent of mGluR activation and protein synthesis but instead requires activation of postsynaptic protein phosphatases (9, 18–20). We examined NMDAR-LTD in *Fmr1*-KO mice to determine whether FMRP selectively regulates protein synthesis-dependent plasticity or LTD mechanisms in general. NMDAR-LTD was elicited by delivering 900 single pulses at 1 Hz (14). In contrast to mGluR-LTD, NMDAR-LTD was normal in *Fmr1*-KO mice (86 ± 4%; 14 slices from 8 animals) as compared with WT littermates (84 ± 4%; 12 slices from 4 animals; P = 0.6; Fig. 3). These results suggests that FMRP may specifically regulate mGluR- and protein synthesis-dependent plasticity.

Discussion

Using two distinct induction protocols, we show that mGluR-dependent LTD is significantly increased in the hippocampus of

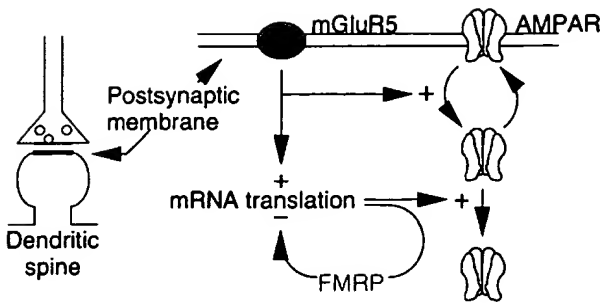

Fig. 4. Model. Previous research has shown that activation of mGluR5 stimulates the internalization of AMPA receptors and NMDARs (not shown; ref. 11). The stable expression of this modification requires protein synthesis, which we propose is negatively regulated by FMRP synthesized in response to mGluR activation. Therefore, in the absence of FMRP, LTD magnitude is increased.

animals lacking FMRP. The most straightforward hypothesis is that FMRP regulates LTD downstream of the mGluRs, likely at the level of mRNA translation.

Although many questions remain, we believe the implications of these data warrant sharing this finding without delay. If aspects of fragile X syndrome are related to exaggerated mGluR-dependent synaptic plasticity, drugs that inhibit group 1 mGluRs and/or LTD might be considered for the treatment of this disorder.

Involvement of FMRP in the Regulation of LTD. Activation of postsynaptic group 1 mGluRs (primarily mGluR5), either by the selective agonist DHPG or by synaptically released glutamate, triggers LTD at Schaffer collateral synapses in area CA1 of the hippocampus. Recent evidence suggests that one expression mechanism for the LTD of synaptic transmission is the internalization of AMPA and NMDARs (11, 21). Both synaptic depression and glutamate receptor internalization can be initiated by mGluR activation without new protein synthesis, but the stable expression of the change fails to occur when mRNA translation (but not transcription) is inhibited (9, 11). The critical site of protein synthesis is the postsynaptic dendrite (9).

One mRNA that is known to be translated in response to postsynaptic group 1 mGluR activation encodes FMRP (6). Thus, the present experiments were designed to test the obvious hypothesis that the mGluR-dependent synthesis of FMRP plays a role in the stabilization of LTD. We were initially surprised to discover that LTD was actually enhanced in the absence of FMRP; however, this finding is consistent with a number of recent studies suggesting that FMRP can function as a negative regulator of mRNA translation (5, 12, 13). Taken together, the data are consistent with a model in which an increase in FMRP normally serves to limit expression of LTD by inhibiting mGluR-dependent translation of other synaptic mRNAs (Fig. 4). One message that is negatively regulated by FMRP encodes the microtubule associated protein MAP1b, which has been shown in *Drosophila* to regulate synaptic structure and function (5). Indeed, recent studies have shown an increase of MAP1b mRNA on polyribosomes in cells derived from fragile X patients, consistent with FMRP being a negative regulator of MAP1b translation (3). It will be of considerable interest to examine the role of MAP1b in the expression of hippocampal LTD.

In addition to LTD triggered by activation of group 1 mGluRs, there is a second well-studied form of homosynaptic LTD that is induced by activating NMDARs (22). In hippocampal slices, expression of NMDAR-mediated LTD is not protein synthesis dependent for at least 1 h (9, 23) and does not occlude mGluR-mediated LTD (10, 17). Our finding of normal NMDAR-LTD in the *Fmr1*-KO mice supports the idea that these forms of LTD use distinct mechanisms. It is interesting to note that another form of NMDAR-dependent plasticity, LTP, is also unaffected in *Fmr1*-KO mice (7, 8). Taken together, the results suggest that FMRP may be selectively involved in synaptic modifications that are triggered in response to mGluR-stimulated protein synthesis.

Role of LTD and FMRP in Cortical Development. It has been suggested that mechanisms of LTD and LTP normally work in concert to fine-tune patterns of synaptic connectivity during development (24, 25) and to store memories in the adult brain (26). One consequence of activating mGluRs in cultured hippocampal neurons is a long-term decrease in the surface expression of the ionotropic glutamate receptors that mediate synaptic transmission, possibly as a prelude to synapse elimination (11). Thus, in the absence of FMRP, enhanced LTD could interfere with the establishment and maintenance of strong synapses required for normal brain function.

In this context, it is noteworthy that dendritic spine development is slowed in the cerebral cortex of *Fmr1*-KO mice (27). Dendritic spines are the major targets of glutamatergic synapses in the cortex. Synapses are formed during development when long thin protospines emitted by pyramidal cell dendrites make contact with nearby axons (28). As the synapse stabilizes, the spines shorten and become fatter. An increased percentage of long thin dendritic processes, reminiscent of protospines, is a characteristic feature of cortical neurons in FMRP-deficient mice (27, 29) and affected humans (30, 31). It was previously suggested that this phenotype might be a consequence of reduced synapse elimination (6). We propose instead that the underlying defect may actually be enhanced activity- and mGluR-dependent synapse turnover, abnormally prolonging a state in which neurons are actively seeking new synaptic input. Consistent with this idea, it was reported very recently that hippocampal neurons in culture express significantly longer thinner spines after DHPG treatment. Like LTD, this effect of DHPG requires protein synthesis (32).

Treatment of Fragile X Syndrome. The intriguing association of group 1 mGluRs and activity-dependent protein synthesis is not restricted to early postnatal development, the cerebral cortex, or LTD. mGluR- and protein synthesis-dependent LTD can still be elicited in hippocampus from mature animals, where it may contribute to memory storage, particularly during novel or stressful situations (33–35). Moreover, recent work has also shown that LTD in the cerebellum, long known to depend on group 1 mGluRs and believed to contribute to learning motor reflexes (36), also requires rapid translation of mRNA (37). Finally, there is evidence that mGluR-triggered protein synthesis in the hippocampus can reduce the threshold for synaptic potentiation (38) and trigger epileptiform activity (39, 40). It is conceivable that FMRP normally functions as a negative feedback regulator of all these physiological processes. In this context, it is interesting to note that the prominent features of fragile X syndrome also include heightened responses to novelty, compulsions, and seizures.

Taken together, the data lead us to hypothesize that fragile X mental retardation is a consequence of increased mGluR-dependent protein synthesis and/or LTD in the brain, both during early postnatal development and in adulthood. We have found that LTD magnitude increases with increasing activation of mGluR5 (10). It follows that titration of a competitive antagonist will produce a graded reduction in this mGluR- and protein synthesis-dependent response. Thus, our hypothesis

prompts an obvious question: Could inhibitors of group 1 mGluR-mediated synaptic transmission be effective in the treatment of this disorder? Although additional studies are obviously required to test this hypothesis, these data point to a rational pharmaceutical approach for fragile X syndrome.

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